Universidade de Aveiro Departamento de Biologia
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Mercury effects in natural populations of sea anemone *Actinia equina*

Efeitos do mercúrio em populações naturais de anémona-do-mar *Actinia equina*

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, ramo de Biologia Molecular e Celular, realizada sob a orientação científica do Prof. Doutor Fernando Manuel Raposo Morgado, Professor Associado com Agregação no Departamento de Biologia da Universidade de Aveiro e co-orientação da Doutora Isabel Maria Cunha Antunes Lopes, Investigadora Auxiliar do Departamento de Biologia e Centro de Estudos do Ambiente e do Mar da Universidade de Aveiro.

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keywords *Actinia equina*, mercury, bioaccumulation, oxidative stress, NMR spectroscopy

abstract

Mercury (Hg) is considered to be one of the most toxic metals to biota, being capable of biomagnification and bioaccumulation in food chains. High concentrations of Hg were associated with developmental and behavioral abnormalities, impairment in growth and reproduction, and reduced survival. Though this metal has been extensively studied, several knowledge gaps still exist, mainly regarding its effects in different types of organisms.

Accordingly, this study aimed at assessing the effects of a short-term exposure to Hg in natural populations of the sea anemone *Actinia equina*. To attain this objective, four natural population of *A. equina* were sampled in the NW Atlantic coast with different levels of contamination for Hg. From each population, forty individuals were collected and acclimated in laboratory, under optimal conditions, for one month. Afterwards, organisms were weighed (7.09 ± 0.18 g mean ± SE), and using a flow-through system were exposed to 100 μg L^{-1} of Hg and to artificial sea water solely, during 96h. At the end of exposure, organisms were immediately deep-frozen (-80°C) until further analysis. The tissue of pedal disc was analyzed for bioaccumulation and biomarkers (biochemical: glutathione S-transferase–GST, catalase–CAT and lipid peroxidation–LPO and metabolic: lactate and alanine) to compare the uptake, oxidative damage and metabolic patterns among populations after being exposed to Hg.

All population exhibited significant bioaccumulation of Hg and a significant change in LPO when exposed to Hg, as in lactate/alanine ratio. On the contrary, exposure to Hg caused no significant changes in the activities of GST and CAT. Furthermore, significant differences in the response patterns to Hg among the four populations were observed. With the two populations originated from sites with a higher Hg contamination showing a lower bioaccumulation of Hg and a higher oxidative damage. Also, the ratio of lactate/alanine (an index of redox state of the cell) revealed that those two populations exhibited a healthier redox state. These findings suggest that the use of several endpoints at different levels of biological organization is important for the evaluation of Hg effects in the biota.

palavras-chave

Actinia equina, mercúrio, bioacumulação, stress oxidativo, espectroscopia por NMR

resumo

O mercúrio (Hg) é considerado um dos metais mais tóxicos para o biota, podendo biomagnificar e bioacumular nas cadeias tróficas. Concentrações elevadas de Hg nos organismos foram associadas a anomalias no desenvolvimento, comportamento, crescimento, na reprodução e a taxas de sobrevivência reduzida.

Embora atualmente este metal seja extensivamente estudado, ainda existem falhas no conhecimento, principalmente no que respeita aos efeitos que pode provocar em diferentes tipos de organismos. Assim, este estudo teve como objetivo avaliar os efeitos de uma exposição, de curta duração, a Hg, em populações naturais da anémona-do-mar *Actinia equina*.

Para atingir este objetivo, foram amostradas quatro populações naturais de *A. equina*, na costa NW Atlântica. Em cada população, foram recolhidos quarenta indivíduos e posteriormente aclimatados em laboratório, sob condições óptimas, durante um mês. Após aclimatação, os organismos foram pesados (7,09 ± 0,18 g, média ± erro padrão), e com a utilização de um sistema de fluxo contínuo, foram expostos a 100 µg L^{-1} de Hg e a água do mar artificial sem adição de Hg, durante 96h.

No final da exposição, os organismos foram imediatamente congelados (-80 °C) até se proceder à análise. O tecido do disco pedal foi analisado para determinar níveis de bioacumulação e efeito a nível de vários biomarcadores (bioquímicos: glutationa S-transferase-GST, catalase-CAT e peroxidação lipídica LPO, e metabólicos: lactato e alanina), de modo a comparar concentrações do metal, danos oxidativos e padrões metabólicas entre as populações após terem sido expostos a Hg.

Todas as populações apresentaram bioacumulação significativa de Hg relativamente ao respectivo controlo, assim como alterações significativas, na LPO e no rácio lactato/alanina. Pelo contrário, a exposição a Hg não causou mudanças significativas na atividade das enzimas GST e CAT. No entanto, foram observadas diferenças significativas nos padrões de resposta a Hg entre as quatro populações naturais de *A. equina.*

As duas populações provenientes de locais com historial de contaminação por Hg mais elevado apresentaram menor bioacumulação de Hg e maior dano oxidativo, em laboratório. Além disso, a proporção de lactato/alanina (um índice do estado redox da célula), revelou que as duas populações apresentaram um melhor estado redox. Estes resultados sugerem que o uso de vários critérios de avaliação em diferentes níveis de organização biológica, são importantes para a avaliação dos efeitos Hg no biota.

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Abbreviations

AAS	Atomic absorption spectrometry
AMA	Advanced Mercury Analyser
ANOVA	Analysis of variance
BBO	Broadband Observe
BHT	Butylated hydroxytoluene
CAT	Catalase
CDNB	1-chloro-2,4-dinitrobenzene
CRM	Certified reference materials
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DW	Dry weight
DTPA	Diethylenetriaminepentaacetic acid
GC-MS	Gas Chromatography-Mass spectrometry
GPS	Global positioning system
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSG	Glutathione oxidized
GST	Glutathione S - transferase
NMR	High-resolution Nuclear resonance magnetic
^{1}H NMR	Proton Nuclear magnetic resonance
INAG	National Institute of Water
LC-MS	Liquid Chromatography-Mass spectrometry
LPO	Lipid Peroxidation
MT	Metallothionein
ROS	Reactive Oxygen Species
SE	Standard error
SOD	Superoxide Dismutase
SPM	Suspended particulate matter
TBA	2-Thiobarbituric acid
TBARS	Thiobarbituric acid-reactive substances
TCA	Trichloroacetic acid
TORT-2	Lobster Hepatopancreas Reference Material
USEPA	United States Environmental Protection Agency
WW	Wet weight

Chapter I

Chapter I: General Introduction and aims of the thesis

1. General Introduction

The high economical importance of coastal zones has resulted in its vast urbanization and industrialization. As a consequence of such an increased human activity, these environments have become contaminated by sewage, industrial and agricultural outfall and accidental spills of different chemicals [1, 2]. This anthropogenic contamination can wield different effects on exposed populations, generating changes in community structure and ecosystem functioning [3]. Within this perspective, the present work focused on the effects of mercury (Hg) contamination on a bioindicator species, because is considered to be one of the most toxic metals to biota, being capable of biomagnification and bioaccumulation in food chains [4]. Though Hg has been extensively studied, several knowledge gaps still exist, mainly regarding its effects in different types of organisms, such as the sea anemone *Actinia equina*. This species has been considered a good sentinel species for monitoring metal pollution in coastal zones [5]. In order to accurately understand the effects that Hg may cause in *A. equina* it is necessary to evaluate several endpoints at different levels of biological organization, such as individual, tissue and subcellular/cellular levels.

To assess such endpoints at different levels of biological organization it is necessary to use a set of several tools in same organism. So, the integrated analyses of biochemical and metabolic biomarkers with the bioaccumulation of Hg may be accurate bioindicators of Hg exposure and give the information of state of stressful of the organism.

1.1. Marine coastal pollution

The marine pollution can have impacts at all levels of biological organization from cellular to ecosystem levels [6, 7]. For example, toxicological challenges due to exposure to pollution may involve impair immune responses and could be responsible for the dramatic increase in the incidence of diseases affecting populations of marine organisms

over the last few decades [8, 9]. Impacts and eutrophication can lead to community level changes, such as shifts in the relative abundance of primary producers and consumers, which may influence primary productivity at an ecosystem scale [8].

For many years we know that evolutionary adaptation to a changing biotic or physical environment depends on available genetic diversity and on natural selection, a concept implicit in most biological studies [10-12]. Natural populations exposed to polluted areas for long periods of time are possibly subjected to selective pressures for an increased resistance to toxicants [13, 14]. This can result in the evolution of resistance, which may have important implications for decisions regarding safe ambient toxicant levels [13]. In fact, it has been argued that a full understanding of ecotoxicological processes must consider an integrated multilevel approach, in which molecular impact is related with higher-order biological consequences at the individual, population and community levels [15].

The fact that organisms have developed resistance to a contaminant may be related to the fact the organisms may have acquired a degree of tolerance by physiological acclimation during exposure to sublethal concentrations at some prior period of their lives. This does not confer tolerance to lethal concentrations upon offspring, who must also be pre-exposed to acquire it. On the other hand the populations may have evolved genetically based resistance, through the action of natural selection on genetically based individual variation in resistance [13].

The majority of polluted areas contain different types of contaminants and this complexity hinders the research in the evolution of resistance, since it makes difficult to pinpoint the selective force [14]. For this reason and given that is more easily to understand the adverse effects that contaminants may have on the biota, the study was centered in only one contaminant. As such we focus our work on the Hg contamination because of its nonessential function in biological organisms, the toxic effects it causes on marine organisms and because of high contamination with data recorded by Hg pollution in the history of the Atlantic coast [16-18].

Among metals, Hg is a priority pollutant due to its persistence, bioaccumulation and toxicity in the coastal zones. It is estimated that atmospheric input of Hg has tripled over

the past 150 years, and that two-thirds of the current total is of anthropogenic origin, while one-third is from natural source. Due to its high toxicity the understanding of the biogeochemical dynamics is, thus, considered of great importance in these ecosystems [19-21].

In marine organisms are highly susceptible to oxidative effects from environmental contamination for Hg, especially when they can generate or enhance the production of reactive oxygen species (ROS), such as Hg [22]. The ROS as a general pathway of toxicity is induced by many redox cycling chemicals (hydrocarbon quinones, nitro-aromatics, biphenyls), transitions metals, and many other compounds leading to conditions of oxidative stress [23, 24]. These ROS consist in superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radical ($OH^{\cdot}$) [23]. To minimize oxidative damage to cellular components, marine and other organisms have developed antioxidant defenses. The chemical defense is comprised of several classes of proteins that function coordinately to protect the cell [23, 25]. Organisms can adapt to increasing ROS production by up-regulating antioxidant defenses, such as the activities of antioxidant enzymes. Failure of antioxidant defenses to detoxify excess ROS production can lead to significant oxidative damage including enzyme inactivation, protein degradation, DNA damage and peroxidation of the lipids [24].

In general, Hg in the aquatic environment tends to be associated with suspended particulate matter (SPM) and is scavenged from the water column to bottom sediments. Sediments may function as repository of anthropogenic Hg or as internal sources when Hg is released to pore water. Transfer of Hg to the water column may deleteriously affect the biota [20, 26-28].

Unlike most other metals, inorganic Hg is efficiently biotransformed into organic forms (e.g. methylmercury), in several compartments of the aquatic environment, including the water column, and both forms (organic and inorganic) can be potentially harmful to biota [29]. Both the organic and inorganic forms of Hg may be transferred from the abiotic to the biotic compartment [30]. Methylmercury is a common seafood contaminant that illustrates the complex interactions between marine pollution, accumulation in food chains, and human health [5, 31]. Due to methylmercury, be the

most toxic and bioaccumulative form of Hg, and have a large capacity for biomagnification in food webs, mostly through food consumption [31]. Even though the mechanisms underlying Hg toxicity are still unclear, its ability to react with and deplete free sulfhydryl groups as well as to disrupt cell cycle progression and/or induce apoptosis in several tissues is well recognized [32].

Bioaccumulation, along with persistence of Hg in environment and toxicity, is used for aquatic environmental hazard identification to determine the potential for adverse effects to biota [33]. The organisms take up and accumulate Hg in soft tissues to concentrations several orders of magnitude above the environmental levels, this bioaccumulation of naturally occurring substances occurs along a continuum of exposure [34].

The bioaccumulation of Hg can occur from sea water, from suspended particles, from sediments and through food chains. The incorporation of this metal in an organism depends not only on the bioavailability of Hg but also on a whole range of biological, chemical and environmental factors [18, 35]. Two particular factors are the biological amplification of Hg along food chains and the acquisition of an increased tolerance to this metal [35]. The concentration of a Hg in an organism is the result of many variables such as the concentration of the Hg in the water, the physical chemical form of the Hg, the membrane permeability of the organism, the type and quantity of food and its degree of contamination, the physiological state of the organism and the characteristics of the physical environment, influencing the organism as well as the metal [36]. Furthermore, the Hg bioaccumulation can be complex, as it is influenced by multiple routes of exposure and geochemical effects on bioavailability [37].

Measuring of Hg in marine organisms may be a bioindicator of Hg exposure on organism and ecosystem health, but the real evaluation of the damage inflicted by this metal should come from comprehensive biomarker studies [38].

1.2. Tools for evaluation of exposure to Hg

The use of several tools for evaluation of exposure of Hg, such as biochemical and metabolic biomarkers and the behavioral responses, together with accumulation, can offer a more complete and biologically more relevant information on the potential impact of Hg on the health of organisms [39, 40].

Biomarkers are defined as quantitative measures of changes in the biological system and give responses at different levels of biological organization, such as biochemical, histological, or physiological alterations or manifestations of environmental stress, in a given organism or population [6, 15, 41]. The use of biomarkers in environmental pollution assessment enables monitoring of stress responses ranging from the biomolecular/biochemical to the population and community levels [42].

The variability of responses existing in biomarkers has been observed and these changes are correlated with different biotic and abiotic factors like temperature, salinity or dissolved oxygen in aquatic ecosystems [6, 43]. But in laboratory studies, organisms are exposed to a selected toxicant under environmentally controlled conditions and in this way we can take into account the response of the biomarkers [43, 44]. This event is not the only one to consider, Lagadic *et al.* [45] explained the importance of measuring several biomarkers at the same time in the same organisms, which allows a pertinent and integrative approach to evaluate the effects of pollutants on individuals. This multiparametric approach using different and/or complimentary biomarkers will enable an assessment of the effects of the different contaminants present in the aquatic environment [15]. The choice of the appropriate biomarker for monitoring chemical exposure requires knowledge of a variety of factors, because a slight change can influence the biomarker response in many invertebrates [6].

The biochemical biomarkers used in this study, are antioxidants, due to the ability of Hg to cause oxidative stress in marine organisms. This research has focused on glutathione S-transferase (GST), catalase (CAT) and lipid peroxidation (LPO), because are often used to evaluate metal contamination [6, 25, 46]. As metabolic biomarkers, we

used the metabolites lactate and alanine that enable to verify the metabolic performance of organisms when exposed to Hg [47-49].

1.2.1. Biochemical biomarkers

Biological systems have developed during their evolution adequate enzymatic and non-enzymatic antioxidant mechanisms to protect their cellular components from oxidative damage. These include antioxidant enzymes, such as superoxide dismutase (SOD), CAT, glutathione peroxidase (GPx), glutathione reductase (GR) and GST, as well as some molecules with antioxidant action such as glutathione, uric acid and ascorbate [50].

1.2.1.1. Glutathione S-transferases

Biotransformation can be defined as a conversion of a xenobiotic substance into a more water soluble forms by enzymes, which can be more easily excreted from the body. Xenobiotics can be biotransformed in highly metabolic organs (e.g. liver, kidney, digestive gland) by enzymes from phase I and II. Phase I is a non-synthetic alteration (oxidation, reduction or hydrolysis) of the original molecule, which can be conjugated in phase II. Generally, the most sensitive biomarkers of effect consists in alterations in the levels and activities of biotransformation enzymes [50, 51]. In the phase II system, the metabolite may be conjugated with various polar endogenous substrates such as glutathione, glucuronic acid, and sulfate, which are covalently added to xenobiotics [52]. The enzymes catalyze conjugation reactions, thus facilitating the excretion of chemicals by the addition of those groups to the xenobiotic or metabolites [50]. The phase II system plays a critical role in detoxification of xenobiotic substance. Glutathione (GSH), in addition to being a substrate of glutathione *S*-transferase (GST), also serves as an important antioxidant function. Induction of antioxidants can provide sensitive early warning signals of incipient oxidative stress [52]. The GSTs enzymes have an important role in defense against oxidative damage and peroxidative products of DNA and lipids. These enzymes are mainly located in the cytosolic fraction [50].

The cytosolic GST, catalyze the conjugation of GSH with compounds having electrophilic centers (nitrocompounds, organophosphates, organochlorines), generating less toxic and more water-soluble products, easily excreted from cells after further metabolism. The toxicity of many exogenous compounds can be modulated by induction of GSTs [6, 53]. These enzymes exist in multiple forms and elevated activities have been associated with resistance to all the major classes of insecticides, and also some metalloids and pesticides [6, 44].

1.2.1.2. Catalase

The CAT is a heme-containing enzyme that facilitates the removal of H_2O_2, which is decomposed to O_2 and water. The biological importance is more evident from various studies due to the fact that H_2O_2 is the main cellular precursor of the hydroxyl radical ($OH^{\cdot}$) which is a highly reactive and toxic form of ROS. The removal of H_2O_2 is an important strategy of marine organisms against oxidative stress [22]. Because CAT is localized in the peroxisomes of most cells and is involved in the fatty acid metabolism, changes on its activities may often be difficult to interpret [50]. Catalase activity appears to be connected along with the GPx activity to combat the oxidant stress exposure [54].

1.2.1.3. Oxidative damage - Lipid peroxidation

Lipid peroxidation is considered to be a major mechanism, by which oxyradicals can cause tissue damage, leading to impaired cellular function and alterations in physicochemical properties of cell membranes, which in turn disrupt vital functions [24, 55]. Some metals and their chelate complexes are implicated in lipid peroxidation and subsequently in the promotion of carcinogenesis [56, 57].

1.2.2. Metabolic biomarkers: patterns of lactate and alanine

Metabolomics is the study of the complete set of metabolites which are context dependent, varying according to the physiology, developmental or pathological state of the cell, tissue, organ or organism [58, 59]. Metabolomics recently focused on the systematic study of metabolic biomarkers, especially the small molecule metabolites, left behind by the cellular processes. Such study of metabolomic profiling has been widely used in drug toxicity, inherited disease diagnosis, functional genomics, and environmental toxicology [48, 60, 61].

The development of metabolomic as an environmental research tool is a great advantage for environmental risk analysis, discovery of new biological insights and for developing environmental system models [47]. Thus, it became clear the importance of this study in the assessment of Hg effects in marine organisms.

There are several techniques that are already established and are frequently applied in metabolomic studies, such as gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), and high-resolution nuclear resonance magnetic (NMR). However, the NMR spectroscopy such as proton nuclear magnetic resonance (^{1}H NMR), is a method which can provide structural information unattainable by other techniques [62]. The ^{1}H NMR spectroscopy is uniquely applicable to measure a wide range of metabolites from organs or tissue extracts to provide valuable biological and biochemical information on the perturbations induced by both endogenous and exogenous factors [48, 63]. Nevertheless, require minimal sample preparation and is a fast and robust technique, which allows a wide range of small molecule metabolites to be measured simultaneously [48, 58, 64].

1.2.2.1. Proton Nuclear Magnetic Resonance

The increase in exploitation of marine organisms to obtain novel products has been coincident with the invention and development of the ^{1}H NMR. Thus this technique rapidly play a key role in the structure elucidation of marine natural products structure

because can detect all proton bearing compounds in a sample [65, 66]. This approach has proven to be highly sensitive for the detection of effects associated with environmental toxins/xenobiotics, in that, metabolic perturbations are often present much earlier than pollutant induced histopathological changes [58]. Moreover, this technique may detect the toxin/xenobiotic itself, but also obtain the concentrations of endogenous metabolites induced by the toxin, xenobiotics or pollutants [67]. So, obtaining a profile of metabolites present in body fluids or tissues of organisms can be compared between healthy individuals and those subjected to a toxic compound [58, 68]. However, few NMR spectral analyses have been performed for the characterization of invertebrates [67]. In this work we proposed, with the help of this technique, to find metabolic biomarkers in sea anemone *A. equina*. For such, we focus in the levels of lactate since it is a metabolite that indicates anaerobic respiration [47-49] and the levels of alanine, because is another important biomarker in many marine organisms. The alanine it has also been reported that this metabolite constitutes the major portion of end product of glucose breakdown anaerobically [69]. These two metabolites provide the largest variation in metabolomes [47-49, 69].

1.2.3. Behavioral responses during exposition to Hg

Behavior arises from the cumulative interaction of a variety of biotic and abiotic factors and represents the response of the animals to internal (physiological) and external (environmental) factors, relating one organism to another [70]. Behavior is adaptable and its type, intensity, and time of occurrence within a genetically based behavioral tolerance range can be modulated, hence behavior represents an important mechanism to react and adapt to environmental changes including exposure to contaminants. To study changes in behavior due to contaminant exposure is therefore an essential part of behavioral science, which can be called behavioral ecotoxicology. As contaminants are one type of environmental stressors, behavioral ecotoxicology can be seen as integral part of stress ecology. Behavioral ecotoxicology studies how behavior is modified by environmental toxicants [70-72].

The effects of Hg on the behavior of marine organisms are investigated for the purpose of developing behavioral tests applicable to routine toxicological assays. In this study we use the behavior responses to observe the effects of Hg in individual levels of biological organization.

1.3. *Actinia equina* as bioindicator species

The sampling sites are typically intertidal rocky shores and are situated in NW Atlantic coast. These habitats are notable for their diversity, mainly marine invertebrates, such as mussels, urchins, barnacles and limpets, sea stars and sea anemones, of several sizes and colors. Typically, are open ecosystems with steep environmental gradients, subject to a wide range of natural fluctuations on various temporal and spatial scales [8, 73, 74]. These peculiar characteristics by itself or in conjunction with anthropogenic pollution events, led these sites to become ecologically important [73, 75].

For evaluation of contamination for Hg in these sampling sites we used a sessile invertebrate, sea anemone *A. equina* (L., 1758), as bioindicator of pollution (fig. 1). The *A. equina* are particularly suited to monitoring studies and have been used for the detection and monitoring of chemical pollution in coastal waters and ecotoxicological tests in laboratory. Also, they have a large distribution that range extends from the sub polar coasts of north Russia (Kola Peninsula), southward along all the Western European coastlines, into the Mediterranean Sea and as far as the west coast of Africa [76-79]. This species exists in a large number of phenotypic variants, which differ from one another considerably in appearance (e. g. red, green and brown, with generally plain, but also striped or spotted columns), choice of habitat and reproductive strategy (sexually or asexually), and whose taxonomic position remains largely unclear [79-82]. The young brooded in the gastrovascular cavity of adults of both sexes, is then released and will attach itself to the adjacent substratum [73, 83]. The color of the brooded young juveniles has generally been observed to be the same as that of the parent [80]. These organisms can glide slowly along the substrate with the help of their pedal discs. The large adhesive area of the pedal disc also confers a strong hold on the rocky surface and makes the anemone extremely difficult to dislodge. After being established in a favorable spot,

an anemone can sit tight and expand/stretch their tentacles in search of small vertebrates and invertebrates, which are then overpowered and carried to their mouth. *Actinia equina* catch the preys during high tides and contract the tentacles during low tides, without having to roam around in search of food. Because of this type of behavior, these organisms are named opportunist omnivorous predators [80, 84, 85].

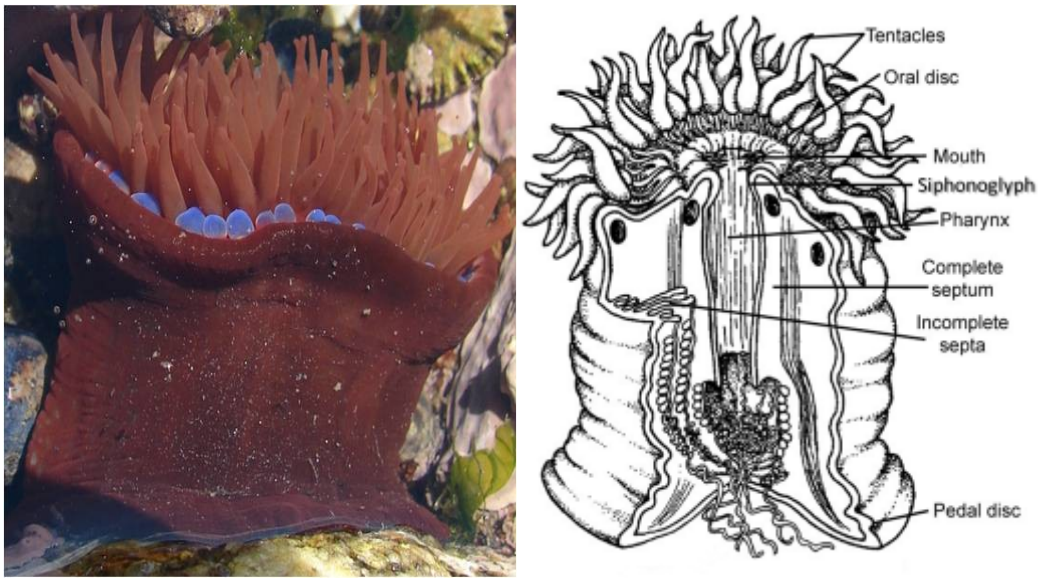

Figure 1. Morphology of *Actinia equina*: A) real representation; and B) schematic representation, according to Graaff and Crawley [86].

When disturbed, sea anemones contract and withdraw their tentacles and oral discs. Some anemones are able to swim to a limited extent by rhythmical bending movements, which may be a mechanism of escaping from predators such as sea stars and nudibranchs [84, 87]. The sea anemones and other organisms of this phylum have specialized cells, called cnidocytes (or nematocyts). These cells are located in several parts of the body, both internal and external, but they are especially abundant in the tentacles, where they serve in predation and defense, and so are extremely essential to their mode of life[84, 87, 88].

1.3.1. Ecotoxicological Relevance

In general, the effect of metals in cnidarians has received relatively little attention, despite the importance of many cnidarians species in coastal communities worldwide [25, 89, 90]. But some few studies have shown that when chemically stressed, these species possess morphological and physiological adaptations to environmental forcing factors, making them an effective bioaccumulation indicator, since they are very common and live in environmental coastal areas which are continually exposed to anthropogenic activity [5, 91].

The long life of sea anemones, may play an important part in the control to retain all the valuable spaces, in contrast to short-lived organisms, whose ecology is dominated by the need for regular replacement by new recruits [73]. Particularly the sea anemones of the genus *Actinia* has become a good model to study not only due to its ecological importance (high levels of genetic variation and high differentiation between geographically close populations), but also because individuals of this genus are small, occur at high densities, are exclusively occupants of hard substrata [92], and are easily collected due to their often conspicuous and almost sedentary nature. In laboratory, they are very simple to maintain [80] and some studies showed the potential of this species to be used as a bioaccumulation indicator of some metals [5, 93].

2. Aims of the thesis

The main goal of this study was to assess the effects that short-term exposure to mercury may pose to natural populations of the sea anemone *Actinia equina*, exhibiting different histories of exposure to this metal. To accomplish the main objective, two specific goals were delineated:

1. To characterize the levels of exposure to Hg of the natural populations of *Actinia equina* at the four sampled sites in the NW Atlantic coast;
2. To assess the effects, at different levels of biological organization, of a short-term exposure to Hg of *Actinia equina* collected at the four sampled sites.

The present thesis is organized in the following Chapters:

Chapter I – General Introduction and aims of the thesis

Chapter II – Characterization of the levels of mercury exposure in four natural populations of *Actinia equina*

Chapter III – Effects of a short-term exposure to mercury at different levels of biological organization in *Actinia equina*

Chapter IV – Conclusion

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Chapter II

Chapter II: Characterization of the levels of mercury exposure in four natural populations of *Actinia equina*

1. Introduction

Mercury is considered to be an environmental pollutant of high priority across the world, mainly due to the high toxicity of many of its forms even at very low concentrations, as well as due to the easy uptake of its organic forms by biota and its biomagnification in the food chain [94]. Although restrictions on anthropogenic emissions have been set recently, Hg buried in sediments may be released to the water column through physical disturbance or changes in the physicochemical environment (e.g. redox potential, temperature, oxygen, salinity), becoming available to aquatic organisms [95]. Mercury occurs in various physical and chemical forms in the natural aquatic environment, including elemental mercury (Hg^0), ionic mercury (Hg^+, Hg^{2+}), and various inorganic and organic forms. The main organic forms being methylmercury CH_3Hg^+ and dimethylmercury $(CH_3)_2Hg$ [96, 97]. The nature and reactions of these species determine the solubility, mobility, and toxicity of Hg in aquatic ecosystems, as well as the potential for methylation [96]. Of these species, the methylmercury is the one that raises more interest in an ecotoxicological point of view, since it is a neurotoxin. In addition, methylmercury tends to bioaccumulate and biomagnify more readily than other species of Hg [97]. Owing to its lipophilic and protein binding properties, methylmercury is readily accumulated by aquatic biota and may thus also pose a threat to humans and other fish-eating animals [96].

For evaluation of Hg effects in marine organisms, the species under research is the common intertidal rocky shores beadlet sea anemone *A. equina*, because exists in a large number of variants and has a wide geographical distribution along the coastal areas in Europe and the Mediterranean, with a strong representation in the Portuguese coast [5, 79, 98]. Therefore, some studies showed the potential of this species as a bioaccumulation indicator for some pollutants [5, 91, 99]. Accordingly, this study aimed to characterize the levels of exposure to Hg in natural populations of *A. equina* at four

sites located in the NW Atlantic coast. As such, concentrations of total Hg have been determined in the two tissues of sea anemone, tentacle and pedal disc. Finally, the concentration of total Hg in water, sediments and suspended particulate was also determined, for environment Hg accumulation comparison with in organisms.

2. Methodology

2.1. Study area

Four study sites were selected in the NW Atlantic coast in order to reflect different degrees of anthropogenic contamination for Hg (fig. 2): S1 – Sanxenxo (N 42º23' 57.0''; W 0.08º 48' 26.8''), S2 – Vila Praia de Âncora (N 41º 49' 13.48''; W 08º 52' 24.13''), S3 - Vila Chã (N 41º18' 11.5''; W 0.08º 44' 16.5'') and S4 – Aguda (N 41º 0.2' 34.8''; W 0.08º 39' 05.4''). The geographical coordinates were determined using a mobile global positioning system (GPS12 Personal Navigator®, Garmin®) in each sampled site. In this northern region, from S1 till S4, the rocky shores are typically granitic, except S3, where rock platforms consist of shales [100, 101]. The most northerly sampled station, S1, is localized in Galicia, Spain. This location forms a bay, downstream of the Ria de Pontevedra in the north zone. The Ria is a coastal embayment of about 20 km long that gets broader in the NE-SW direction [102]. There is some evidence of anthropogenic induced enrichments in the Ria, such as some metals (Cu, Zn, Hg, Pb) [103]. The site S2 is located to the south of the Minho River, where have been detected petroleum derived products (fluvial traffic) and urban effluents [104]. This site is characterized by a vast rocky shore and a small beach, which forms a sandy inlet [105]. Site S3 is located 25 km North of Oporto and some small population aggregates exist near the beach with reduced contamination sources. Human activities in the area are mainly small-scale traditional agriculture and fishery [106-108]. Finally, site S4 is located 15 km south of the river Douro estuary and, given the proximity to this site it may have receive influences from the estuary, in which have been detected indices of chemical pollution [109].

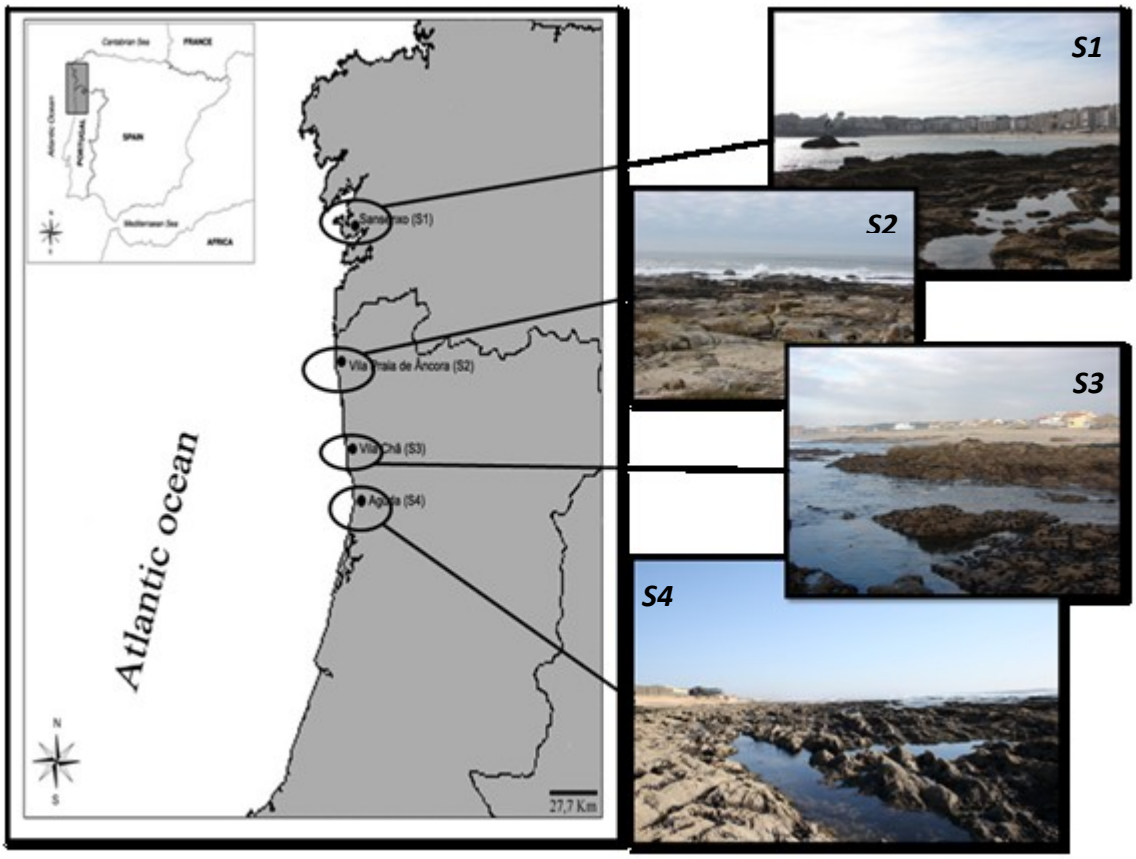

Figure 2. Map with the localization of the sites where the four populations of *Actinia equina* were collected, in the NW Atlantic coast: S1 – Sanxenxo, S2 – Vila Praia de Âncora, S3 – Vila Chã, S4 – Aguda.

2.2. Sampling procedures

At each sampling site, the following physicochemical parameters were measured at sub-surface water samples: temperature, dissolved oxygen (DO), pH, salinity, Hg concentrations and suspended particulate matter (SPM). These measurements were made with the VWR® Symphony ™ meter in the field, with exception of SPM and Hg concentrations. To measure this parameter, a volume of 10 L of water sample was collected, transported to the laboratory in plastic bottles and immediately filtered using pre-weighted 0.45 µm pore size HA filters (Millipore). Filters were re-weighted after heating, overnight at 40 °C and stored at 4 °C, until analysis. The concentration of SPM was determined by the ratio between the mass of dry material retained in the filter and the volume of water filtered [20, 50]. For total dissolved Hg analysis, the filtrates obtained in the separation of dissolved and particulate phases were collected and acidified with a HNO_3 solution, separated in three replicates, and stored in a refrigerator at 3-4 °C until

analysis [20]. Then the particulate total Hg (Hg in SPM, mass of Hg in SPM per volume of water) was also analyzed.

Surface sediments were also collected in all the sampled sites, in the top 3 – 5 cm, near the sea anemones habitat. Sediments were dried during three days at 40 °C using a stove. After drying, sediments were homogenized and sieved for coarse (1000 – 63 μm) and fine (< 63 μm) fractions. The samples were kept at room temperature until analysis of the total Hg.

Finally, at each site, fifty individuals of *A. equina* were collected at the intertidal band of the rocky shores in the low tide, during the months of January to March of 2011. Individuals of the species *A. equina* were removed from the rocks using a small metal spatula and the collection was extensive and random. Seven of organisms were immediately stored at -20 °C for posterior analysis of its total Hg content. The remaining individuals were transported in plastic buckets containing natural seawater and maintained in laboratory under optimal conditions until being used for toxicology testing (please see Chapter III for further details).

2.3. Biological samples procedures

Seven specimens of *A. equina* were dissected and tissue samples separated in tentacles and pedal disc. Samples were weighted, freeze-dried with Flexi-Dry MP Freeze Dryer, weighted again and the dry weight (dw)/fresh weight ratio was calculated.

2.4. Total mercury analysis

The filtered water, sediments, SPM and sample tissue of seven organisms of *A. equina* [110], from the four sampling sites were measured for total Hg by atomic absorption spectrometry (AAS). The system allows for the thermal heating of the sample to decompose the sample and release Hg, which is concentrated in a gold amalgam. Thermal desorption of the Hg from the amalgam is detected by AAS using the Advanced Mercury Analyzer (AMA) LECO 254. The entire analytical procedure was validated by replicate

analysis of certified reference materials (CRM). The CRM used was TORT-2 (Lobster Hepatopancreas Reference Material for Trace Metals, National Research Conseil national Council Canada), thereby ensuring that the instrument remained calibrated throughout the study. The obtained values of mean and standard deviation were 0.27 ± 0.01 μg g^{-1} (N=4), and certified values are 0.27 ± 0.06 μg g^{-1}.

2.5. Statistical procedures

In the concentration analysis of total Hg of the sea anemone *A. equina*, a two-way analysis of variance (ANOVA) was carried out to compare the four sampled sites and the two tissues (tentacle and pedal disc), using SigmaPlot® 11 Software. When significant differences were detected (p<0.05), the Holm-Sidak post hoc test was performed to determine the specific differences between mean values. The normality and homoscedasticity of data was verified with the Saphiro-Wilk test.

3. Results and Discussion

3.1. Environmental characterization

Table 1 shows the values of the physicochemical parameters that were measured in the sub-surface water samples collected at the four study sites (S1 to S4).

Table 1. Physical and chemical parameters measured in water sampled of the four sampling sites: S1- Sanxenxo, S2- Vila Praia de Âncora, S3- Vila Chã and S4 – Aguda. Temperature (T), dissolved oxygen (DO), pH, salinity, and suspended particulate matter (SPM).

Site	T (°C)	DO (mg L^{-1})	Salinity	pH	SPM (mg L^{-1}) (mean ± SE)
S1	16.10	30.62	35.30	8.52	11.28 ± 1.02
S2	14.20	10.40	25.00	7.51	10.94 ± 1.69
S3	14.10	9.90	29.10	8.14	18.50 ± 1.25
S4	17.20	9.78	35.50	8.87	14.98 ± 0.70

The temperature ranges from 14.10 °C (S3) to 17.20 °C (S4), and in S1 and S2 are 16.10 °C and 14.20 °C, respectively. Salinity showed variation ranged from 25.00 psu (S2) to 35.5 psu (S4), and S1 and S3 presents the 35.30 psu and 29.10 psu, respectively. The concentration of DO, as shown similar values between 9,78 mg L^{-1} to 10,40 mg L^{-1}, except for S1 which presents the high concentration, 30,62 mg L^{-1}. The concentration of SPM ranges from 10.94 mg L^{-1} to 18.50 mg L^{-1}. The highest concentration was found in S3 and the lowest in S2. The others sites S1 and S4, have intermediate values of concentration (11.28 mg L^{-1} and 14.98 mg L^{-1}, respectively). In general, the sampling sites are similar in their physicochemical parameters.

3.2. Total mercury in seawater

Aiming the study of Hg contamination in sampled sites, it is essential to identify the metal in compartments such as the water column, the sediments and the biota. In the water column, it is important to differentiate the dissolved fraction from the particulate fraction. The former is defined operationally as the fraction constituted by all the forms of

the metal that are not retained by a 0.45 μm pore size filter. In opposition, the SPM is defined as the material retained by the same filter [111].

The concentrations of dissolved metals in seawater are affected by a number of processes, namely biological uptake, scavenging by particulate matter, release from bottom sediments, advection and mixing of water masses [17]. The table 2 shows the concentration of total Hg for the sampled sites, filtered water, suspended particulate (dissolved and particulate fractions of Hg, respectively), and sediments.

The concentration of total Hg in the filtered water (dissolved fraction) varied between 0.16 μg L^{-1} (S1) and 0.71 μg L^{-1} (S2). The low Hg concentration in sites S1 and S4 was probably due to the fact that Hg is transported in association with SPM or by the formation of complexes with the various types of polysulphide that precipitated to the sediment [104]. This seems to be supported by the relatively high concentrations found in SPM (438.62 μg L^{-1} and 304.20 μg L^{-1} of Hg, in sites S1 and S4, respectively).

The comparison of measured concentrations of dissolved Hg in the four water samples, with maximum values established for marine water in guidelines of Europe, United States of America and Australia, suggest that all sampled sites (S1, S2, S3 and S4) in the NW Atlantic coast, can be considered zones with contamination of Hg: National Institute of Water (INAG) standards for superficial water (0.07 μg L^{-1}); United States Environmental Protection Agency (USEPA standard) for marine water (0.14 μg L^{-1}); and Assessment Levels for Soil, Sediment and Water guideline of department of Health Western Australia for marine water (0.1 μg L^{-1}).

The spatial distribution of total Hg concentrations at the surface of the sediments varied between 0.018 μg g^{-1} (S1) and 0.070 μg g^{-1} (S2). Sediments from S4, have concentrations of Hg of 0.040 μg g^{-1} and in those of S3 fine fraction (<63 μm) was not found.

The concentration of total Hg associated with the suspended particles (particulate fraction) ranged from 174.37 μg L^{-1} (S2) to 438.62 μg L^{-1} (S1).

Table 2. Concentrations of total Hg measured in filtered water, SPM (dissolved and particulate fractions), and sediments, collected at the four sampling sites. ND = Not Determined.

Site	$[Hg]_{dissolved}$ (µg L^{-1}) (mean ± SE)	Sediments (< 63 µm) (µg g^{-1}) (mean ± SE)	$[Hg]_{SPM}$ (µg L^{-1}) (mean ± SE)
S1	0.16 ± 0.01	0.018 ± 0.004	438.62 ± 1.50
S2	0.71 ± 0.02	0.070 ± 0.012	174.37 ± 0.90
S3	0.46 ± 0.01	ND	271.34 ± 1.10
S4	0.24 ± 0.02	0.040 ± 0.001	304.20 ± 1.25

Mercury that is introduced into natural waters is rapidly and efficiently scavenged by the fine-grained suspended particles. However, Hg partition between the dissolved and particulate phases is highly variable and dynamic, changing due to several factors and conditions [4]. Also it is well known that sediments may function as net sinks for Hg, due to the binding of Hg to particles with organic coatings and/or precipitated as sulphides. Nevertheless, because of perturbations at the sediment–water interface (i.e. tidal and wind stirring, freshwater flows and bioturbation), contaminated sediments can be resuspended and dispersed within the water column [95].

In sampled sites S1 and S4, the Hg distribution for the three phases was similar, because both sites present the lowest concentrations of total Hg in water and sediments, and to the high values in SPM. In S2 the Hg levels pattern was opposite, since the total Hg concentration in water and sediments was high compared with particulate fraction. Similar conclusions could not be drawn relatively to site S3, as the fine fraction was not found.

3.3. Total mercury in *Actinia equina*

The concentrations of total Hg in tissues of *A. equina* are shown in table 3. Values ranged from 0.0040 µg g^{-1} to 0.0160 µg g^{-1} for tentacles and 0.0030 µg g^{-1} to 0.0110 µg g^{-1} for pedal disc. No significant differences were observed in the measured levels of Hg between the two tissues (tentacle and pedal disc) (table 4).

Table 3. Average concentrations of total Hg measured in two tissues (tentacle and pedal disc) of *Actinia equina* individuals collected at the four sampling sites.

Site	Tissue	[Hg] µg g^{-1} (mean ± SE)
S1	Tentacle	0.0160 ± 0.0028
	Pedal Disc	0.0110 ± 0.0029
S2	Tentacle	0.0040 ± 0.0003
	Pedal Disc	0.0030 ± 0.0002
S3	Tentacle	0.0040 ± 0.0002
	Pedal Disc	0.0030 ± 0.0002
S4	Tentacle	0.0100 ± 0.0031
	Pedal Disc	0.0100 ± 0.0023

Table 4. Results of a two-way ANOVA testing for differences in Hg concentrations in the tentacles and pedal disc of *Actinia equina* individuals collected at the four sampling sites. The statistically different values are marked with asterisk (*), $p < 0.05$. DF = degrees of freedom; SS = sum of squares; MS = mean of squares.

Source of variation	DF	SS	MS	F	*p*
Site	3	350725.179	116908.393	3.695	**0.018***
Tissue	1	82.652	82.652	0.00261	0.959
Site x Tissue	3	106936.796	35645.599	1.127	0.348
Residual	48	1518845.364	31642.612		
Total	55	1976589.990	35938.000		

Concentrations of total Hg was higher in S1, followed by S4, because this similarity the two sampled sites form one group. But the S4 site is not sufficiently distinct from S2 and S3 to be completely isolated from these (fig. 3). There is statistically significant difference between sites ($F = 3.695$; $df = 3, 55$; $p = 0.018$). These results showed distinct behaviors between populations, which may be due to several factors such as, the availability of Hg in the environment and different rates of Hg assimilation by organisms.

The concentration of total Hg found in tissues of *A. equina* suggests a relation with Hg particulate fraction found in SPM. The increased concentrations of Hg in particulate fraction correspond to sites where there was an increase of Hg in the tissues. This event can occur due to the suspensivore behavior of these organisms since the suspended particles are crucial link for chemical constituents between the water column, bed sediment and food chain [112, 113].

Weltens *et al.* [114], suggested that the suspended particles can decrease toxicity of many chemicals for some aquatic organisms by decreasing the free concentration of the molecules, but at the same time they can increase the bioavailability for other organisms.

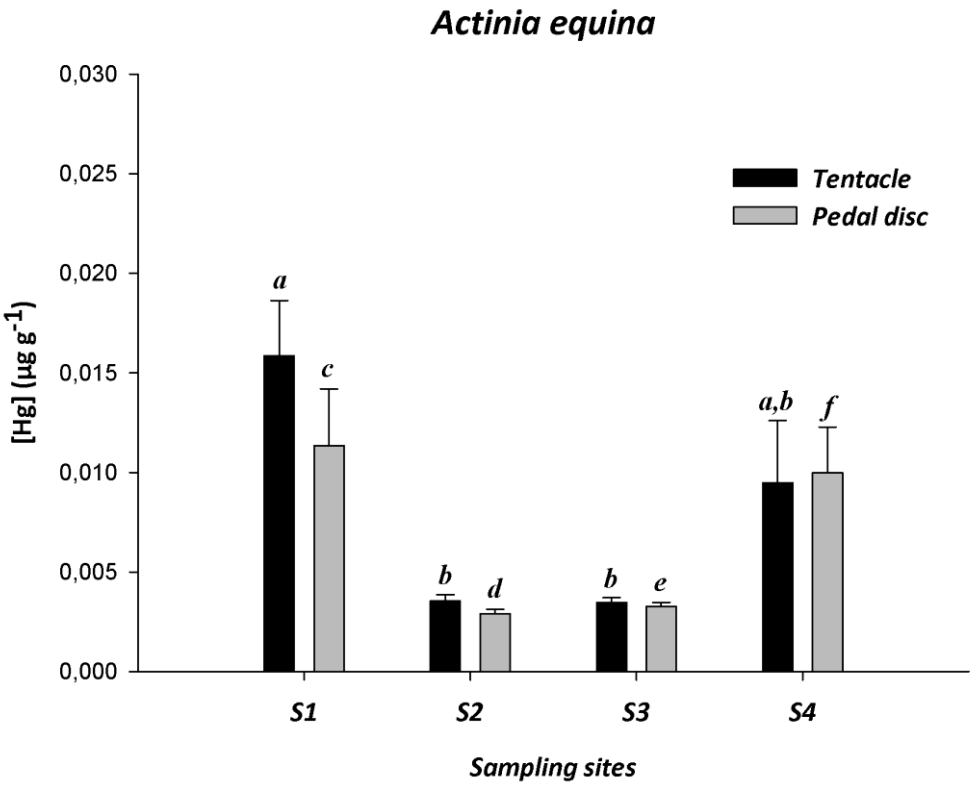

Figure 3. Concentration of Hg in two tissues (tentacle and pedal disc) of *Actinia equina* (µg g^{-1}), collected at four sites in the NW Atlantic coast. The values represent the mean of seven specimens with the corresponding SE bars. Letters *a* and *b* indicate significant differences between sampling sites and letters *c*, *d*, *e* and *f* indicate no significant differences between tentacle and pedal disc, at $p < 0.05$.

The differential concentration of Hg in *A. equina* form the four sampled sites, may be related with others environmental factors. For example, the beaches of S2, S3, and S4 sites receives direct influence of ocean currents and therefore, intense local hydrodynamics when compared with population of S1 that form a bay, this fact can contribute to the highest bioaccumulation of Hg in this site, due to the disturbance induced by wave action. Due to the wave activity and tidal currents causes more expansion the tentacles of *A. equina* and disturbance has been considered important in the distribution of this species. It may act to increase food supply, aid the removal of noxious waste materials [115].

The values found in biota at all sampled sites, when compared with the limit value in Europe, are very low. The limit value is set at 0.5 μg g^{-1} of wet weight (ww) (except for some species, for which it is raised to 1 μg g^{-1} ww) [116].

Table 5 shows the levels of total Hg in some studies. The concentrations vary considerably and can be attributed to hydrogeochemical characteristics and/or contamination of the various systems. In this study, total dissolved Hg concentrations in water of S1 were higher than those observed in other sites of Galicia coast, but total Hg in sediments and in biota were much lower. The sampled sites S2, S3 and S4 showed values for total dissolved Hg very similar with other stations in the NW Atlantic coast, but the levels of total Hg in biota are different [17]. The values for total Hg found in *A. equina* varied considerably in comparison with other organisms on related sites. Gadelha *et al*. [5], for the same species present values much higher of Hg in Santiago de Compostela and Malaga than those measured in this study, and the same was observed for sediments. These differences may be due to the bioavailability of Hg in environment and the different biotic and abiotic factors of each site, and also the occurrence of precipitation and storms during the winter can contribute to the differences found.

Table 5. Levels of total Hg measured in several matrixes collected in aquatic systems located, along the NW Atlantic coast.

Site	Sample	$[Hg]_{total}$	Reference
Coast of Galicia	Seawater	1.0-73.5 ng L^{-1}	[117]
	Sediments	0.050-2.73 µg g^{-1}	
	Mussels	0.101-1.11 µg g^{-1} dw	
Matosinhos, Oporto	Seawater	0.40 µg L^{-1}	[17]
	Enteromorpha spp.	0.16 µg g^{-1} dw	
	Porphyra spp.	0.24 µg g^{-1} dw	
Madalena, Oporto	Seawater	0.32 µg L^{-1}	
	Enteromorpha spp.	0.11 µg g^{-1} dw	
	Porphyra spp.	0.085 µg g^{-1} dw	
Cortegaça, Oporto	Seawater	0.27 µg L^{-1}	
	Enteromorpha spp.	0.094 µg g^{-1} dw	
	Porphyra spp.	0.075 µg g^{-1} dw	
Santiago de Compostela, Spain	Sediments	0.149 µg g^{-1}	[5]
	A. equina	0.6 µg g^{-1}	
Malaga			
	Sediments	0.8 µg g^{-1}	
	A. equina	3.8 µg g^{-1}	
Pontevedra, Spain	Mussels	0.09-0.29 µg g^{-1} dw	[118]

The toxicity of Hg to marine organisms is affected by both biotic and abiotic factors, namely by the environmental conditions (e.g., temperature, salinity and pH), the sensitivity of different species of organisms to the metal, the life history stage and the tolerance of individual organisms [111].

Gadelha *et al*. [5], explained the process of Hg uptake in *A. equina*, stating that uptake occurs mainly via ingestion of contaminated food. This can occur during sea anemone life cycle depending on the diet, water conditions and individual susceptibility for environmental contaminants. These statements corroborate the data obtained in the present study, since the highest values of Hg in the tissues correspond to organisms that belong to the same sites where the highest values of contamination was found in the particulate fraction.

At all studied sites, the concentration of Hg was always higher in sediments or water than in organisms. This indicates that *A. equina* species might have an efficient

depuration system, since previous studies reported that these are useful organisms for metal biomonitoring in aquatic environment [5]. Those observed differences may be explained due to the intra-specific variability existent in the patterns of accumulation, including regulation of body concentrations of some metals by some species, and the vastly different concentrations present in the environment [37]. Other study suggest that sea anemone *Anthopleura elegantissima*, after an initial lag period, may have up-regulated metals efflux mechanisms or some mechanism for clearing these metals [90]. This information might be of great value for studies of bioaccumulation/depuration of Hg in different tissues of sea anemone and for the mechanisms involved in this process, since these mechanisms for Hg accumulation, differential uptake and removal may exist in the common sea anemone *A. equina*.

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Chapter III

Chapter III: Effects of a short-term exposure to mercury at different levels of biological organization in *Actinia equina*

1. Introduction

In the last years, research works have shown that metals are able to disturb the integrity of several mechanisms in marine organisms [119]. Among metals, Hg is of special concern mainly due to its ability to react with and deplete free sulfhydryl groups as well as to disrupt cell cycle progression and/or induce apoptosis in several tissues [32]. Mercury is capable of inducing oxidative stress in marine organisms [24, 120], this stress results from the production of ROS, and has gained considerable interest in the field of ecotoxicology. It has also been suggested that oxidative stress biochemical biomarkers could be employed in environmental monitoring programs [22, 120] and the formation of ROS has been pointed out as a key mechanism in Hg-induced toxicity [32].

In ecotoxicological research a powerful tool to measure the effects of Hg in organisms is the use of several techniques, because it provides insights in several metabolic pathways simultaneously. Due to the highly complex nature of the metabolic pathways, the assessment of the metabolic status of an organism can never be based on the measurement of a single biomarker [121]. For example, for some species the measurement of contaminants accumulating in tissues, the use of several biomarkers and the ecotoxicological behavior, can offer a more complete and biologically more relevant information in the potential impact of contaminant to organisms health [40, 122].

The exposure to Hg can induce a chain reaction of biochemical processes at the (sub)cellular level aimed at restoring the metabolic equilibrium which, if not achieved, might lead to effects at higher levels of biological organization [121]. So, the aim of this work was to assess the effects, at different levels of biological organization, of a short-term exposure to Hg in *A. equina* collected at the four sites described in Chapter II.

2. Methodology

2.1. Maintenance and experimental conditions

The organisms collected at the four sites described in Chapter II, were acclimated to laboratory conditions for one month. The objective of this acclimation period was to eliminate the environment influences, to reduce inter-individual differences among the experimental testing, to allow the elimination of any previously taken up chemicals, and permit the stabilization of the organisms at the laboratory. The organisms were maintained in aquaria (15 x 33 x 13 cm) filled with artificial seawater, under continuous aeration, approximately six organisms were introduced per aquarium. Circulating and filtered seawater was provided to the aquarium with a skimmer and biological and physic filters in a flow-through system, at approximately 20 °C ± 1 °C in a temperature controlled room. The organisms of each site were maintained in separated seawater tank, but the total conditions remained the same. The salinity was maintained at 35 psu, pH at 7.5 ± 0.5, dissolved oxygen level was 8.4 ± 0.2 mg L^{-1}, and the photoperiod of 14:10h of light:dark cycle (fig. 4).

The organisms were fed twice each week (Advanced Fish Feed ZM-400), with an amount of food proportional in about 2 % of their weight [85]. The seawater medium was composed of tap water to which commercially available sea salt (Ocean Fish, Prodac), was added. Once a week the evaporated water was replaced by desalinated water, and 10 % of the total volume was changed by newly mixed seawater solution [76].

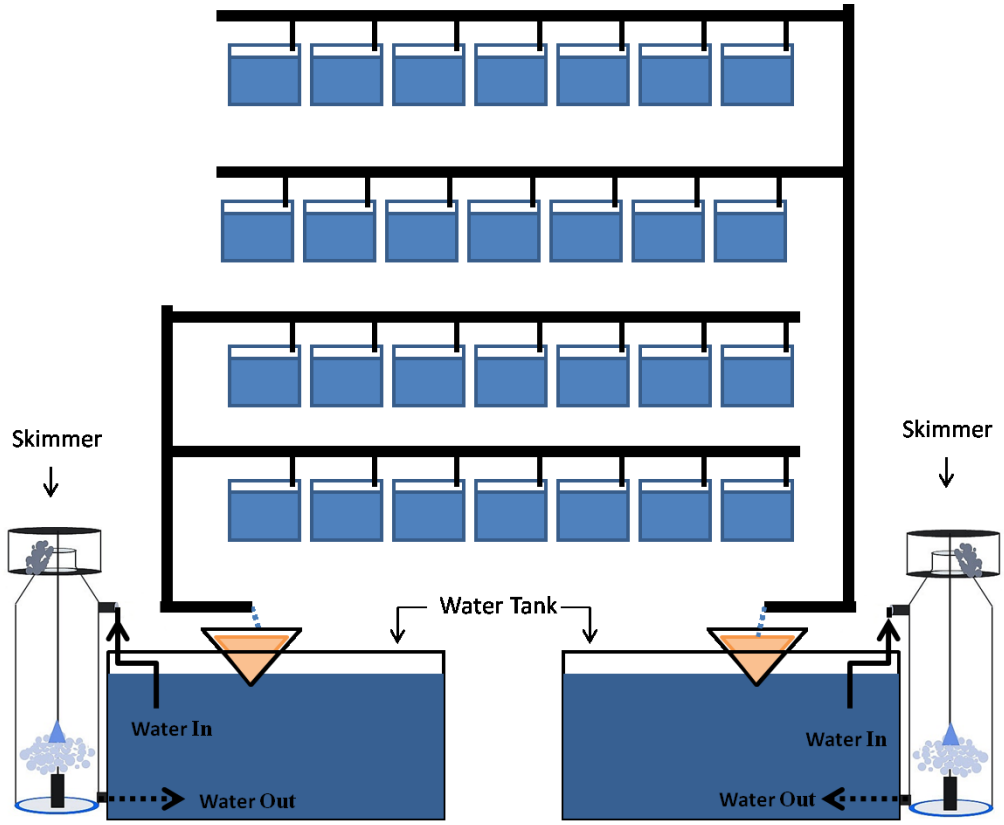

Figure 4. Schematic representation of the laboratory acclimatization system: flow-through system with circulating and filtered synthetic seawater.

2.2. Short-term exposure

The short-term exposure to Hg during 96h was performed using standard methods [110]. From each site, only the organisms exhibiting expanded tentacles were chosen to carry out the short-term exposure to Hg. The wet weight and morphological description of all organisms that were used to perform the test (N=160) were recorded, the mean (± standard error) wet weight being 7.09 ± 0.18 g. Each sea anemone was carefully separated from the aquarium of acclimatization and was introduced in the vessel of the system test. The test was performed in identical physicochemical conditions as those set for acclimatization. In brief the system was flow-through and closed, and each sea anemone was maintained in vessels of 1 L. The water quality (pH, DO and salinity) of test system was monitored throughout the experiment, daily.

The systems were built with the same seawater medium, each containing a separate tank, with the following treatments: control (no Hg added), with 15 animals of each site; and contaminated with 100 µg L^{-1} Hg with 25 animal of each site. We selected these concentrations as non-lethal based on previous bioassays with other aquatic organisms (table 6) [119, 123, 124].

Table 6. Values of the median lethal concentrations (LC50) and median effective concentrations (EC50) for Hg determined for several aquatic species.

Species	Endpoint		[Hg]	Reference
	LC50	EC50		
Porites astreoides	**72h**		**37 – 180 µg L^{-1}**	[123]
Freshwater fish	**96h**		**33 – 400 µg L^{-1}**	[125]
Enchytraeus albidus	**21 days**		**23.5 – 28.6 mg Kg^{-1} dw**	[126]
Paronychiurus kimi	**7 days**		**3.9 mg Kg^{-1} dry soil**	[127]
		28 days	**0.23 mg Kg^{-1} for lead**	
Carcinus maenas		**96h**	**154 – 340 µg L^{-1}**	[124]
Early larval stages:				
Palaemon serratus		**72h**	**74 µg L^{-1}**	[128]
Maja squinado		**72h**	**72 µg L^{-1}**	
Homarus gammarus		**48h**	**48 µg L^{-1}**	
Pomatoschistus microps		**96h**	**62 µg L^{-1}**	[119]
Psetta maxima		**96h**	**62.3 – 12.5 µg L^{-1}**	[129]
Lemna minor		**96h**	**640 µg L^{-1}**	[130]

To obtain these concentrations, we used a stock solution of 10 mg L^{-1} mercury chloride ($HgCl_2$, Sigma Aldrich). Due to the number of organisms used and liters of water needed, the short-term exposure was realized in two phases: in the first phase the test was carried out with two populations, Vila Praia de Âncora (S2) and Vila Chã (S3); in the second phase the test was performed with Sanxenxo (S1) and Aguda (S4). The Hg was

administered directly to the tank at each daily seawater renewal, because the results of the preliminary test, showed that concentration of Hg was lost after 24h.

Each test in the contaminated system contained 55 L in total (0.80 L x 50 vessels + 15 L tank), and the control system contained 34 L in total (0.80 x 30 vessels + 10 L tank), and the flow-through was 18 ml/min (fig. 5).

At the end of the exposure (96h), the organisms were frozen in liquid nitrogen and kept at -80 °C, until analysis, and the water samples were taken to measure the actual metal concentrations.

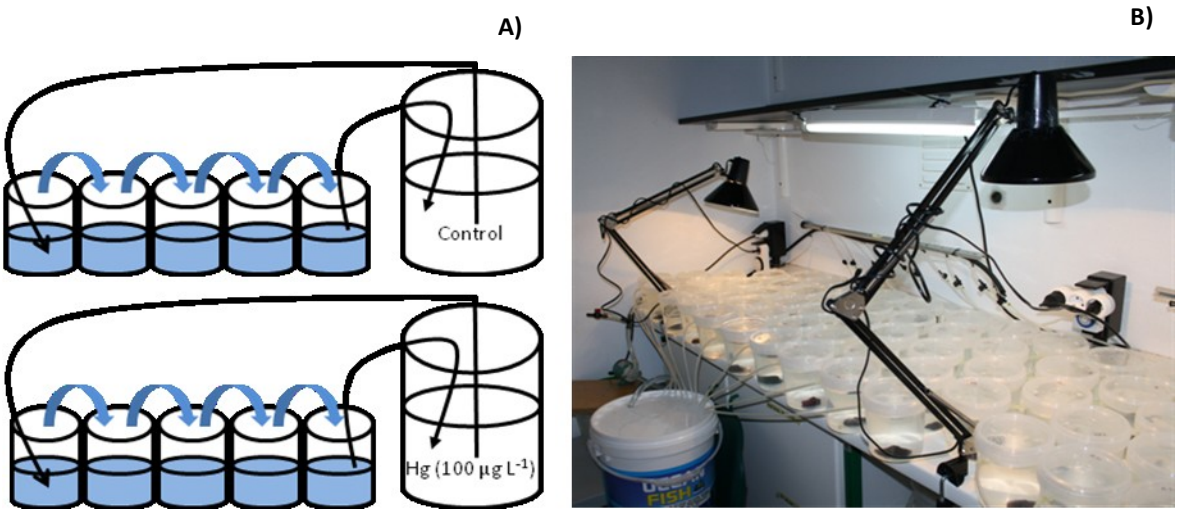

Figure 5. Flow-through and closed test system: A) scheme of the mode of water circulation, for the control and Hg treatment, separately; B) image of de flow-through system in laboratory.

2.3. Behavior responses

During the bioassay, the behavior of the organisms was observed every day at the same hour (1 P.M.) (analysis at the level of the organism): 1) the position of the organism in the vessel of the test was noted and divided into four categories: center, edge, corner and side (fig. 6); 2) the observations of the oral disc open were categorized on a scale from one to five, one being closed and five being opened, methodology according to DiMarco [131] (fig. 6); 3) finally, the number of the juvenile polyps were also recorded.

After 48h of acclimatization in system test these observations were recorded (T0), and after the starting of the test the other times were recorded at 24h (T1), 48h (T2), 72h (T3) and 96h (T4).

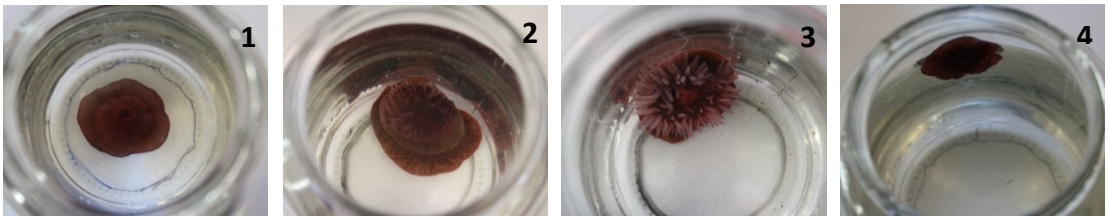

Figure 6. Images illustrating the position of *Actinia equina* during the short-term exposure to Hg in the test vessel. Four categories were defined: 1) center position, 2) edge position, 3) corner position and 4) side position. Observations of oral disc open were recorded on a scale from one to five, one being retracted and five being expanded: 1) Total retracted stage 1; 3) Total expanded stage 5.

2.4. Biological samples procedures

After the short-term exposure, all animals were analyzed for bioaccumulation of Hg (tissue level), and biochemical biomarkers (cellular/subcellular level). For metabolic biomarkers five replicates of each condition were separated for analyzing with the ^{1}H NMR spectroscopy (subcellular level). For several analysis the tissue used was the pedal disc, because in the field is the part of sea anemone that is in direct contact in the environment, not only substrata but also water current. Thus receives the influence of several environmental compartments. In the laboratory remained the same principle, since gastrovascular cavity is the region that control metabolic processes of the organisms, such as absorption, digestion or excretion and the pedal disc is connected with this tissue, we can easily measure the activity of the enzymes GST, CAT and LPO (described below) [132-134].

The samples used for the determination of bioaccumulation of Hg, were weighted, freeze-dried with Flexi-Dry MP Freeze Dryer, weighted again and the dw/fresh weight ratio was calculated. For the other two techniques (chemical and metabolic biomarkers), the samples were used without this previous treatment.

2.5. Mercury analysis of tissues

The samples of pedal disc of *A. equina*, from the all sampled sites, were measured for total Hg by atomic absorption spectrometry (AAS). The system allows for the thermal heating of the sample to decompose the sample and release Hg, which is concentrated in a gold amalgam. Thermal desorption of the Hg from the amalgam is detected by AAS using the Advanced Mercury Analyser (AMA) LECO 254. The entire analytical procedure was validated by replicate analysis of CRM. The CRM used was TORT-2, thereby ensuring that the instrument remained calibrated throughout the study. The obtained values of mean and standard error were 0.218 ± 0.001 μg g^{-1}, and certified values are 0.270 ± 0.060 μg g^{-1}.

2.6. Biochemical biomarkers analysis

For the measurement of the biochemical biomarkers, samples were sonicated (Kika Labortechnik, V200S control, Germany), in 0.1 M potassium-phosphate buffer, pH 7.4, 1 ml per 200 mg of tissue, to release the enzymes from the cells. From the homogenate, 300 μl were separated to a microtube and 2.50 μl of 4% butylated hydroxytoluene (BHT) in methanol was added for endogenous LPO determination. The remaining tissue homogenate was centrifuged at 10.000 g for 20min (4 °C) to isolate the post-mitochondrial supernatant. All samples were stored at -80 °C until posterior protein determination, GST and CAT activity analysis.

Glutathione S-transferase (EC 2.5.1.18) was determined by following the increase in absorbance at 340 nm due to the formation of the conjugate 1-chloro-2,4-dinitrobenzene (CDNB) (substrate)/GSH, catalyzed by GST, according to the method of Habig *et al.* [135]. A volume of 100 μl of supernatant was mixed in 200 μl of a reaction solution. The reaction solution was a mixture of 4.95 ml K-Phosphate 0.1 M (pH 6.5) with 900 μl GSH 10 mM, and 150 μL CDNB 10 mM. It was measured at 340 nm. The enzymatic activity was expressed as units (U) per mg of protein and calculated as nmol of substrate hydrolyzed per minute, using a molar extinction coefficient of 9.6 $mM^{-1}cm^{-1}$.

Catalase (EC 1.11.1.6) was determined based on the method described by Clairborne [136]. A volume of 50 µl of supernatant was mixed with 500 µl H_2O_2 0.03 M, and 950 µl K-Phosphate 0.05 M (pH 7.0) and absorbance was recorded spectrophotometrically at 240 nm, by measuring the decomposition of the substrate H_2O_2. The enzymatic activity is expressed as unit (U) per mg of protein, and corresponds to one µmol of substrate hydrolyzed per minute, using a molar extinction coefficient of 40 $M^{-1}cm^{-1}$.

The determination of LPO was performed in tissue homogenate, according to the procedure of Bird and Draper [137] and Ohkwa *et al.* [138], by measuring thiobarbituric acid-reactive substances (TBARS) at 535 nm. The reaction included a mixture of 300 µl homogenized tissue (initially separated), 1 ml trichloroacetic acid (TCA) 12% (w/v), 1 ml 2-thiobarbituric acid (TBA) 0.73% (w/v) and 800 µl Tris-HCl 60 mM with diethylenetriaminepentaacetic acid (DTPA) 0.1 mM. The reaction mixture was then incubated at 100 °C in a water bath for 1h. After this, tubes were centrifuged for 5min. at 11.500 rpm (25 °C). Samples were kept away from light, at 25 °C and immediately read at 535 nm. Lipid peroxidation is expressed as nmol TBARS hydrolyzed per minute per g of ww using a molar extinction coefficient of $1.56x10^5$ $M^{-1}cm^{-1}$ [43]. All the biochemical determinations were performed in a Multiskan® Spectrum.

2.7. Protein quantification

The protein concentration was determined according to the Bradford method [139], adapted from the BioRad Bradford micro-assay set up in a 96 well flat bottom plate, using bovine γ-globuline as protein standard.

2.8. Statistical procedures

The statistical analysis of data was done using two-way analysis of variance (ANOVA), which was carried out to compare the two condition in the test (control and exposed to Hg) and the individuals of the four sampled sites. When interactions were significant ($p <$

0.05), Holm-Sidak post hoc test was performed to determine the specific differences between mean values. Behavior data was tested using the Manny-Whitney test and the significance of results was ascertained at $p < 0.05$. The graphs and statistical were completed using SigmaPlot® 11 Statistics. All data are preset in this work using the values mean and the corresponding standard error (mean ± SE).

2.9. Proton Nuclear Magnetic Resonance spectroscopy

2.9.1. Tissue extracts

The frozen tissues were pre-cooled in liquid nitrogen and pulverized to a fine powder under liquid nitrogen using a pestle and mortar. Methanol and chloroform (4 °C) in a ratio of 2:1 (v/v; 3 ml/g tissue) were added to the frozen ground tissue and the tissue–solvent mixture was allowed to thaw, before being sonicated during 15min at 4 °C. After sonication, chloroform and distilled water were added to the samples in a ratio of 1:1 (1 ml/g tissue) to form an emulsion. The samples were then centrifuged at 12.000 *g* for 20min. The upper phase (methanol and water) was separated from the lower (organic) phase and freeze-dried overnight. The resulting powder was then dissolved in deuterium oxide (D_2O) for further NMR analysis.

2.9.2. ^{1}H NMR spectra acquisition and analysis

^{1}H NMR spectra of the collected samples were acquired at 9.4 T (Tesla), 25 °C, using a Bruker Avance III 400 spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany) equipped with a 5 mm Broadband Observe (BBO) probe with a z-gradient. Solvent-suppressed ^{1}H NMR spectra were acquired with a sweep width of 6 kHz, using a delay of 14s to allow total proton relaxation, a water pre-saturation of 3s, a pulse angle of 45°, an acquisition time of 3,5s and at least 64 scans. Before Fourier transform, each free induction decay was zero filled and multiplied by a 0.2 Hz lorentzian.

The following metabolites were determined whenever present (multiplet, δ, ppm) lactate (doublet, 1.33), alanine (doublet, 1.45) and H1-α glucose (doublet, 5.22). Sodium fumarate was used as an internal reference (6.50 ppm) to quantify metabolites in solution. The relative areas of ^{1}H NMR resonances were quantified using the curve-fitting routine supplied with the NUTSpro NMR spectral analysis program (Acorn NMR, Inc, Fremont, CA) [140].

3. Results and Discussion

The concentration of Hg used was in sublethal doses, this concentration was determined based in the effects reported in the literature for others aquatic organisms. The short-term exposure of *A. equina* to Hg resulted in significant effects at different levels of biological organization, which is consistent with the initial hypothesis that sublethal toxicant concentration can cause effects in this species.

3.1. Behavior of *Actinia equina* during short-term exposure

Visual observations were made during the course of the experiments. The observations are separated in three categories: oral disc exposure, position in test vessel and number of expelled juvenile polyps by the gastrovascular cavity. All these categories were reported as stressful signaling or typical habits in the field [76, 113, 115, 131, 133].

3.1.2. Oral disc open

For results of observations of oral disc open of *A. equina* during short-term test, the data gathered at all observation periods (T1, T2, T3 and T4) were compared with T0, both for the control and Hg treatment, separately. In the control, and for the four populations, no significant differences in the percentage of organisms with the oral disc opened was registered along of test. In organisms exposed to Hg, with exception of S4 population, all change their behavior along of the test, showing a decreased of organisms with oral disc opened, with significant differences. The percentage of organisms with oral disc opened in the control or exposed to Hg, for T0 and T4, are shows in figure 7. The S2 and S3 populations have more organisms with oral disc opened in the control and when exposed to Hg, in the beginning to the test, when compared with populations S1 and S4. The control of S4 (T4), do not presented organisms with oral disc open. However, in all populations, the organisms when exposed to Hg tend to close the oral disc. So, *A. equina*

showed be sensitive to Hg in the water solution, since changed its behavior during the test when compared with organisms in the control.

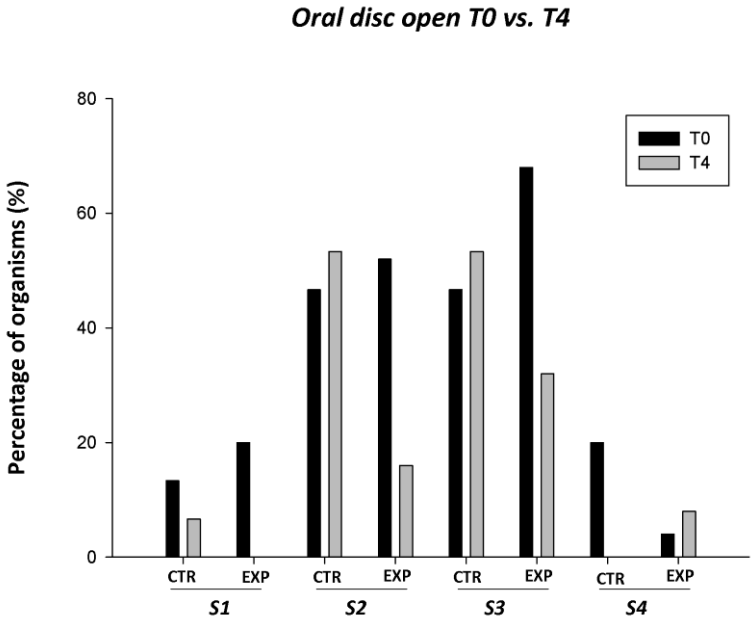

Figure 7. Percentage of individuals of *Actinia equina* with the oral disc totally expanded (stage 5) in the control and when exposed to Hg, at the beginning T0 (00h) and at the end T4 (96h) of the short-term test. S1, S2, S3 and S4 correspond to four populations of *Actinia equina* sampled in the NW Atlantic coast.

3.1.3. Position in the test vessels

For results of position of the organisms in the test vessel the data gathered at all observation periods (T1, T2, T3 and T4) were compared with T0, both for the control and Hg treatment, separately. There was no difference across the treatments (control and exposed to Hg), at each observation time, with exception of population of S4 that show statistically differences starting in the time T2.

Concerning the position of organisms in the vessel test, it was observe that organisms of the control and exposed to Hg, showed a tendency to be positioned at the edge or on the side of the bottle test (fig. 8). In the field, this specie is found in wave exposed zones. Correlating with this fact, the position in the test vessel where current was more intense is its central area. So, the position of the organisms in the vessel is more probably related

to the zone where the current of water is stronger rather than water contamination by Hg.

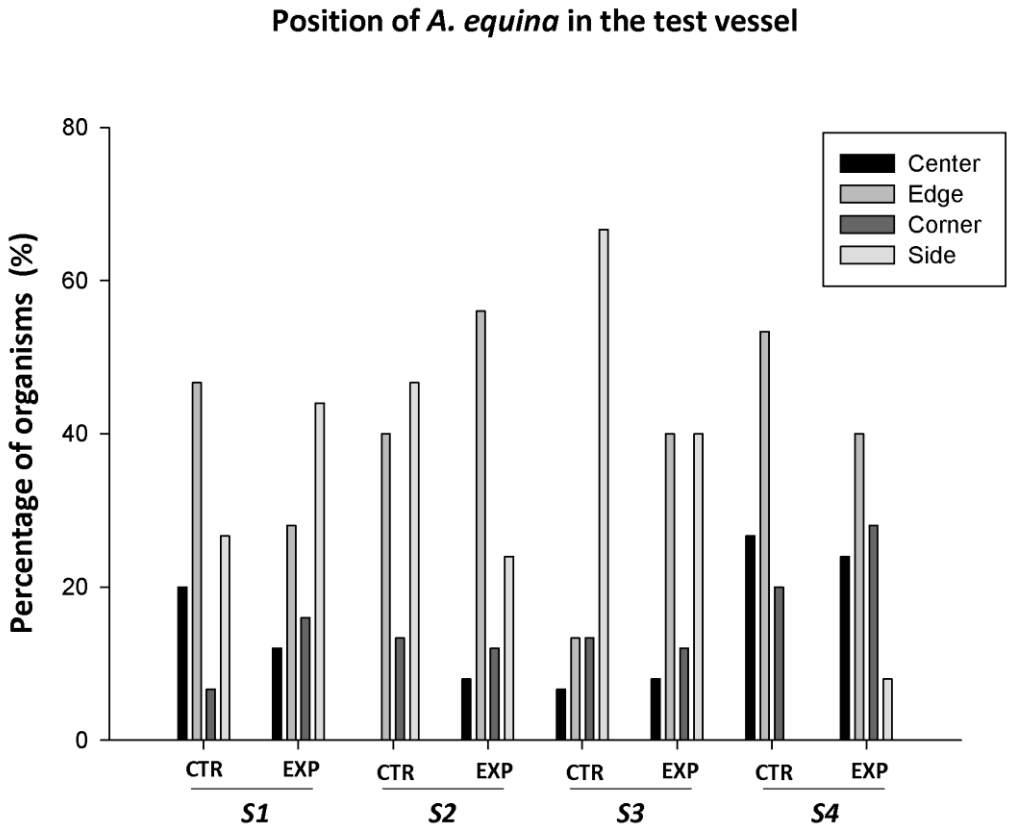

Figure 8. Percentage of individuals of *Actinia equina* which are in four different positions (center, edge, corner and side) on the vessel test, when exposed to Hg at 96h (T4). S1, S2, S3 and S4 correspond to four populations of *Actinia equina* sampled in the NW Atlantic coast.

3.1.4. Reproduction

The reproductive biology of *A. equina* is of interest because this anemone broods its young within its gastrovascular cavity until they are released, usually as small juveniles [73, 83]. Ormond and Caldwell [113], report that reproduction was affected by chronic oil pollution under laboratory conditions, as well as, changes in temperature or lack of oxygen. For these reasons, the increasing the number of young premature freed was used as a response to stressful conditions.

For results of the polyps released during the short-term test, the number of juveniles in the end of experiment were compared to T0, for control and Hg exposed, separately. Only population of S2 site showed significant differences in the end of test, in both treatments. More young were freed in this site than other sampled sites. The others populations were not statistically different along the short-term test. While compared the behavior responses of organisms of the control and exposed to Hg also do not showed statistical differences along of the test.

The *A. equina* when in adverse conditions tend to release the juvenile polyps. Changing organisms from the acclimatization system to the test system was probably sufficient to release the juvenile polyps. For this reason, when the organisms were exposed to Hg, there were no more juvenile polyps inside the gastrovascular cavity or the quantity is much smaller and does not show statistical differences. However, in the S2 population, statistical significance was obtained since more polyps were released during the test than in the other populations. Perhaps the explanation could be that this population is more stressed than the other three populations of sea anemones.

3.2. Bioaccumulation of mercury

In the bioaccumulation of Hg there were significant differences between treatments (control and exposed) and populations (table 7).

Table 7. Results of a two-way ANOVA testing for bioaccumulation of Hg in *Actinia equina* individuals by sampled site and by treatments (control vs. exposed to Hg). The statistically different values are marked with asterisk (*) $p < 0.05$; (**) $0.05 < p < 0.01$; (***) $p < 0.001$. DF = degrees of freedom; SS = sum of squares; SS = sum of squares; MS = mean of squares.

Sources of variation	DF	SS	MS	F	*p*
Bioaccumulation of Hg					
Treatment	1	503.012	503.012	94.613	**<0.001*** **
Site	3	146.438	48.813	9.181	**<0.001*** **
Treatment x Site	3	146.042	48.681	9.156	**<0.001*** **
Residual	145	770.895	5.317		
Total	152	1665.406	10.957		

The organisms from sites S2 and S3, exposed to Hg, accumulated the metal at a higher rate than organisms from sites S1 and S4, under the same exposure conditions (fig. 9). In control, de values of Hg were similar among organisms from the different sites, and ranged from 0,017 μg g^{-1} (S3) to 0.024 μg g^{-1} (S1). At all populations significant differences in the levels of Hg were detected between control and Hg exposed organisms.

In animals exposed to Hg, the values ranged from 0.862 μg g^{-1} to 4.097 μg g^{-1}, these results showed high variability between populations of *A. equina*. In the populations of S1 and S4 sites, the organisms had the lowest concentration (1.405 μg g^{-1} and 0.862 μg g^{-1}, respectively), and in the populations of S2 and S3 sites the highest concentrations were observed (3.773 μg g^{-1} and 4.097 μg g^{-1}, respectively).

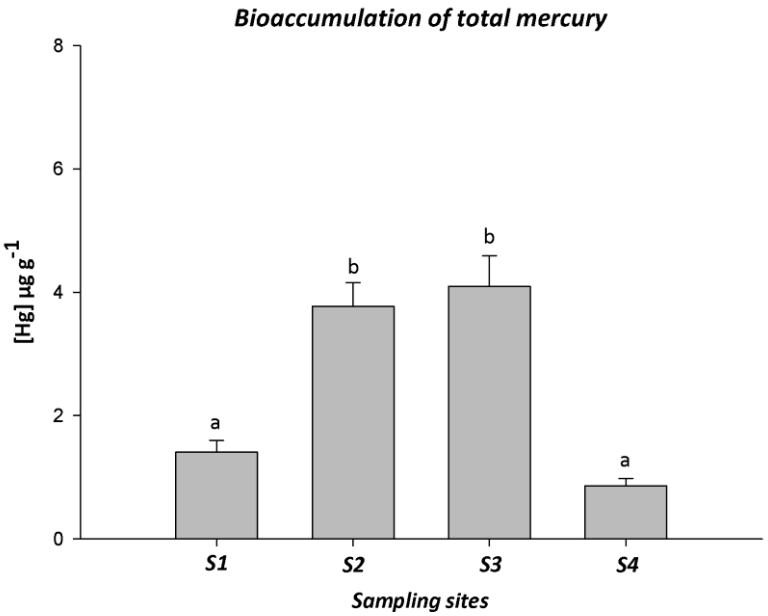

Figure 9. Average of the concentration of Hg in populations of *Actinia equina* (μg g^{-1} dw) that were short-term exposed to 100 μg L^{-1} of Hg. S1, S2, S3 and S4 correspond to four populations of *A. equina* sampled in the NW Atlantic coast. The values represent the mean of all organisms of the test, with the corresponding SE bars. Letters *a* and *b*, indicate significant differences of bioaccumulation between populations, at $p < 0.05$.

The variations between organisms of different sites may be due to the fact that S2 and S3 sites exhibited a higher percentage of organisms with oral disc opened when exposed to Hg when compared with S1 and S4 sites. It is expected that organisms with

the oral disc more opened will have more uptake of Hg by the gastrovascular cavity, which might explain the great variability among organisms.

Considering the historical exposure to Hg of the organisms in the field, S1 and S4 came from sites more contaminated than organisms of S2 and S3, other hypothesis arise and may explain these differences. The differences of Hg concentration found in organisms of the different sites may be due to different rates of metals adjustment inside their bodies. Harland and Ngando [133], explained the possibility that cnidarians are also able to regulate metals, by processes involving the absorption, excretion and storage. Van Praet [132] described the existence of granules in *A. equina* that could be involved in mechanisms of control of metals, although it has not been demonstrated that they have a regulatory function. There have been few attempts to localize metals in relation to the physiology of cnidarians [141]. Mitchelmore *et al.* [90] suggested that the sequestration of metals by granules may act in conjunction with mucus production such that metal granules are concentrated in mucus and discarded.

A few studies have shown that several genera (e.g. *Hydra, Anthopleura*), accumulate metals in tissue and that species exhibit differential sensitivity to particular metals [90, 122, 142]. Andersen *et al.* [143], previously report that sensitivity to metals in cnidarians may be due to the lack of metallothionein (MT), proteins that bind to metals, or other metal binding proteins which have an important role in homeostatic control and sequestration of metal ions. This study has shown that these proteins are absent from *Hydra*, suggesting that it may be absent in all cnidarians too. Later, other research with the species *Nematostella vectensis*, report the absence of members of the evolutionarily conserved metal-binding genes, the class I and II metallothioneins, which are found in diverse eukaryotes, including sponges [144].

Hypotheses on the bioaccumulation of metals in cnidarians have emerged. As an example, Mitchelmore and co-workers, in studies performed on anemone *Anthopleura elegantissima* suggested that the bioaccumulation may be influenced by glutathione, a metal-binding antioxidant, as well as by endosymbionts [89]. Reitzel *et al.* [144] reported results that can corroborate the previous hypothesis, because when searching for homolog proteins known to be involved in sensing stress in *Nematostella* genome, he

detected a number of GPx and GST, although is unknown if this genus could harbor endosymbionts.

3.3. Oxidative stress and peroxidative damage

Marine organisms exhibit a variety of changes in enzymatic antioxidant defenses after the exposure to pollutants with oxidative potential. Viarengo [145] report that some metals can change the activity of several enzymes by binding to their functional groups or by displacing the metal associated with the enzyme. These changes can cause damage in cells. To minimize this oxidative damage to cellular components, organisms have developed antioxidant defenses [24]. There are several important enzymes involved in the mechanism of detoxification of organic pollutants. In this study, GST and CAT were chosen, as these enzymes have been reported as good biomarkers in response to metals [32, 54]. For observed oxidative damage the LPO was measured due to the fact that oxidation of polyunsaturated fatty acids are an important consequence of the oxidative stress [32]. The precise cellular localization of antioxidant enzyme activity in cnidarians in unknown, and can only be speculated from research of other phyla [146].

Levels of GST can be modified by a large range of xenobiotics and also by abiotic factors. Due to their involvement in the detoxification processes of xenobiotics, the GST activity was proposed as a biomarker for several aquatic species such as fishes, crustaceans or molluscs.

In this study, the results of GST activity showed significant differences between organisms collected at the four sampling sites after being exposed to Hg (table 8).

Table 8. Results of a two-way ANOVA testing for levels of GST in the study of *Actinia equina* by sampled site and by treatments (control vs. exposed to Hg). The statistically different values are marked with asterisk (*), when a significant interaction was observed ($p < 0.05$). DF = degrees of freedom; SS = sum of squares; MS = mean of squares.

Sources of variation	DF	SS	MS	F	*p*
Glutathione S-transferase					
Treatment	1	152.218	152.218	0.784	0.377
Site	3	532.671	177.557	0.915	0.435

Treatment x Site	3	1664.179	554.726	2.859	**0.039***
Residual	145	28135.650	194.039		
Total	152	30250.733	199.018		

In animals exposed to Hg, the mean values (± SE) of GST activity ranged from 57.22 ± 2.69 (S3) to 63.84 ± 2.23 (S4) U/mg protein (fig. 10). In the lowest activity of this enzyme was reported for organisms from sites S2 and S3, (58.70 ± 3.03 and 57.22 ± 2.69 U/mg protein, respectively). Organisms collected at sites S1 and S4 exhibited the highest activities of GST (59.46 ± 1.95 and 63.84 ± 2.23 U/mg protein, respectively). Only organisms from site S4, show significant differences between treatments, the organisms exposed to Hg exhibit an increased of GST activity when compared with control.

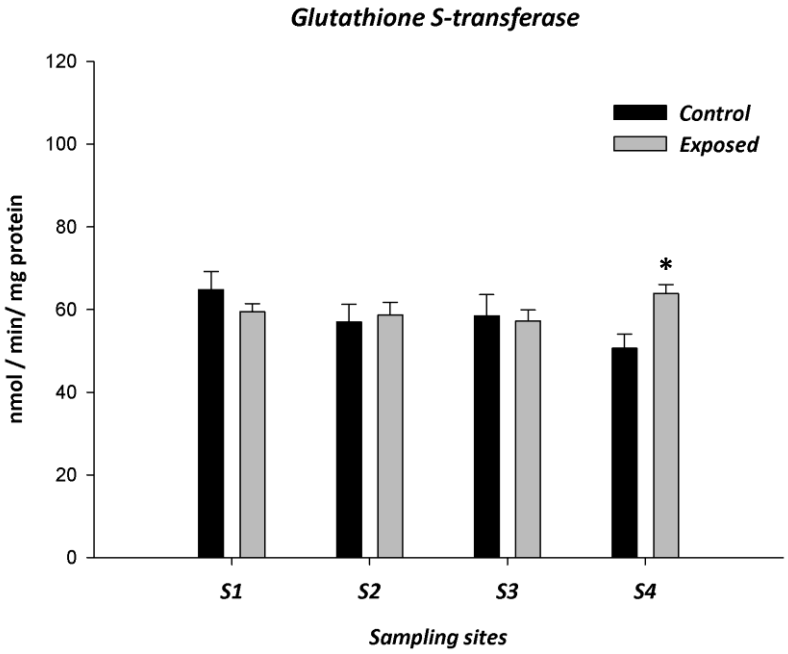

Figure 10. Average of the activity of glutathione S-transferase (error bars represent the standard error) in *Actinia equina* individuals, originated from the four study sites, after being exposed for 96h to artificial sea water and 100 µg L^{-1} of Hg. The statistically differences between treatments (control and exposed to Hg), are marked with asterisk (*), $p < 0.05$.

The decrease of activity of GST in organisms exposed to metals, has been described for several species, like for example in mussels (M*ytilus galloprovincialis*) [147] and in cabbage looper moth (*Trichoplusia ni*) [148]. However, in Castro *et al*. [149], effects on liver GST in the fish *Poecilia reticulata* and *Gambusia holbrooki* were not detected after an

in situ exposure to an acid mine drainage impacted effluent containing a complex mixture of metals. The depletion of GSH in Hg-exposed organisms seems to be, generally, accompanied by a variation in the GST activity [124, 150]. These apparently contradictory results may be due to the time of exposure, the concentration of stressors, and different species [150].

The CAT activity showed significant differences among organisms collected at the sampling sites, however, no significant differences were observed between treatments (control and Hg exposure) (table 9).

Table 9. Results of a two-way ANOVA testing for levels of CAT in the study of *Actinia equina* by sampled site and by treatments (control vs. exposed to Hg). The statistically different values are marked with asterisk (*) $p < 0.05$; (**) $0.05 < p < 0.01$; (***) $p < 0.001$. DF = degrees of freedom; SS = sum of squares; MS = mean of squares.

Sources of variation	DF	SS	MS	F	*p*
Catalase					
Treatment	1	4060.109	4060.109	2.321	0.130
Site	3	30976.357	10325.452	5.904	**<0.001*** **
Treatment x Site	3	12043.729	4014.576	2.295	0.081
Residual	135	236111.308	1748.973		
Total	142	290880.868	2048.457		

In animals exposed to Hg, the mean values (± SE) of CAT activity ranged from 67.78 ± 11.47 to 108.65 ± 4.41 U/mg protein, for (S2 and S1), respectively. Organisms from S2 and S3 sites, exhibited the lowest activities of CAT (67.78 ± 11.47 and 76.04 ± 10.46 U/mg protein, respectively), while organisms from sites, S1 and S4 showed the highest activities of this enzyme (108.65 ± 4.41 and 78.03 ± 8.21 U/mg protein, respectively) (fig. 11).

Only organisms from site S2, show significant differences between treatments, the organisms exposed to Hg exhibit an increased of CAT activity when compared with control. Probably, in these organisms the Hg exposure, triggered an increase of ROS and led to an increased of CAT activity, since it is an important enzyme in antioxidant defense system from oxidative stress [151].

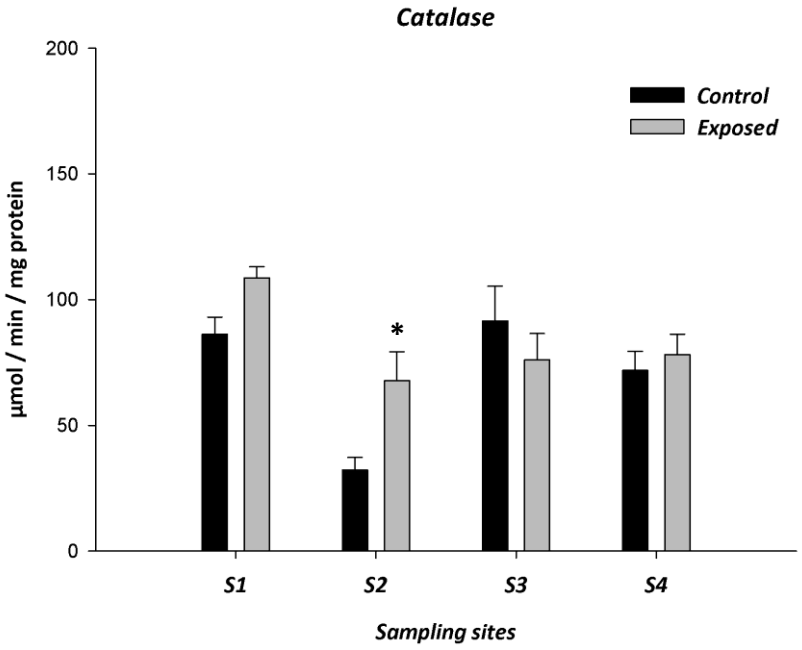

Figure 11. Average of the activity of catalase (error bars represent the standard error) in *Actinia equina* individuals, originated from the four study sites, after being exposed for 96h to artificial sea water and 100 µg L^{-1} of Hg. The statistically differences between treatments (control and exposed to Hg), are marked with asterisk (*), $p < 0.05$.

Lipid peroxidation is one of the main manifestations of oxidative damage and has been found to play an important role in the toxicity of many xenobiotics. The potential consequences of the peroxidative process of membrane lipids include loss of polyunsaturated fatty acids, decreased lipid fluidity, altered membrane permeability, effects on membrane-associated enzymes, altered ion transport, release of material from subcellular compartments, and the generation of cytotoxic metabolites or lipid hydroperoxides [152].

Significant differences were observed in LPO in organisms exposed to Hg when compared with control, and among organisms collected at all sampling sites (table 10).

Table 10. Results of a two-way ANOVA testing for levels of LPO in the study of *Actinia equina* by sampled site and by treatments (control vs. exposed to Hg). The statistically different values are marked with asterisk (*) $p < 0.05$; (**) $0.05 < p < 0.01$; (***) $p < 0.001$. DF = degrees of freedom; SS = sum of squares; MS = mean of squares.

Sources of variation	DF	SS	MS	F	*p*
Lipid peroxidation					
Treatment	1	43900.643	43900.643	21.363	**<0.001*** **
Site	3	167240.944	55746.981	27.127	**<0.001*** **

Treatment x Site	3	22445.798	7481.933	3.641	**0.015***
Residual	130	267150.430	2055.003		
Total	137	523379.908	3820.291		

In animals exposed to Hg, the mean values (± SE) of LPO ranged from 85.49 ± 9.58 to 182.90 ± 9.65 U/ ww, for S4 and S1, respectively (fig. 12). Organisms from S2 and S4 sites, exhibited the lowest LPO (89.57 ± 12.29 and 85.49 ± 9.58 nmol TBARS hydrolyzed per minute per g of ww, respectively), while organisms from sites S1 and S3, showed the highest LPO (182.90 ± 9.65 and 94.45 ± 10.34 nmol TBARS hydrolyzed per minute per g of ww, respectively). In organisms of the all sites, with exception of the S4, a prominent increase of LPO was noted when organisms are exposed to Hg, when compared to organisms in control. With the results obtained we could assume that Hg change the metabolism of the lipid cell membrane components.

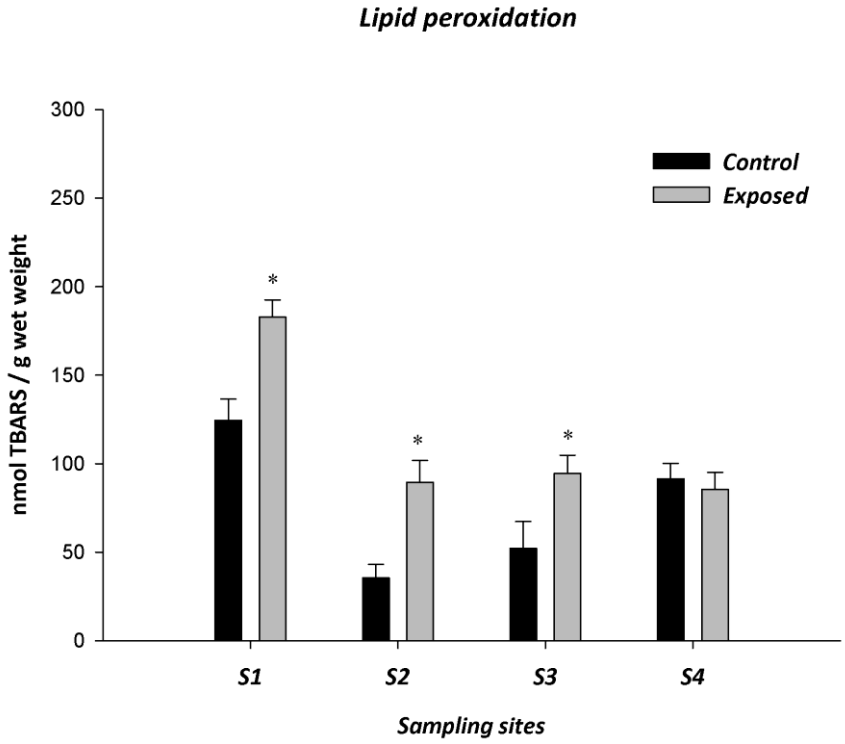

Figure 12. Average of the lipid peroxidation (error bars represent the standard error) in *Actinia equina* individuals, originated from the four study sites, after being exposed for 96h to artificial sea water and 100 µg L^{-1} of Hg. The statistically differences between treatments (control and exposed to Hg), are marked with asterisk (*), $p < 0.05$.

In general, it was not obtained significant differences in antioxidant enzymes when organisms are exposed to Hg comparatively to the control. With exception the organisms of sites S4 (GST activity) and S2 (CAT activity), while showed statistical differences between treatments, these organisms may exhibited more sensitivity to Hg than organisms of S1 and S3 sites. These results can be an indicator that the concentration of Hg used was not sufficient to cause differences between treatments, in organisms of sites S1 and S3.

The organisms of S4 do not exhibited increase of oxidative damage when exposed to Hg, may be related with the increased of GST activity, this antioxidant enzyme may be acting in order to protecting the cells. The organisms of sites S1, S2, and S3 showed oxidative damage when exposed to Hg when compared with control. It is possible that measurement of GST and CAT activity were not sufficient to shown oxidative stress in cells, or the time exposure and concentration of the Hg were not sufficient to observe changes in theses enzymes. The result obtained for LPO may be related with influences the natural environment, since the all sampling sites showed contamination for Hg. The organisms with higher values of LPO in the control came from the sites where animals were found exposed to higher levels of Hg. Therefore, organisms in continuous exposure to Hg tend to exhibit higher levels of oxidative damages.

For complementing the assessment of effects of Hg in *A. equina*, may be important the analysis of the activity of other biomarkers, such as the SOD and GR, and the non enzymatic biomarkers GSH and glutathione oxidized (GSSG). The use of several biomarkers might explain in detail what happens to organisms when exposed to Hg.

3.4. Metabolic pattern of lactate and alanine – ^{1}H NMR spectroscopy

The cells of *A. equina*, when exposed to Hg, may trigger complex redox homeostatic mechanisms to deal with the possible over-production of ROS during oxidative stress. Oxidative damage caused by metals can be explained by changes in the redox state [153]. Therefore, after analysis of biochemical biomarkers for evaluation of oxidative damage in

organisms, we quantified some metabolites to observe the changes in the metabolic and redox state of the cells.

This work has focused in the glycolysis, because this pathway is considered a reliable indicator for biological stress in some invertebrates [47-49], and because aerobic and anaerobic pathways of energy production in sea anemones are similar to others animals [154, 155]. The lactate/alanine ratio is an index of redox state of the cell as the reduction of pyruvate into lactate or its conversion into alanine is related with the $NADH/NAD^+$ ratio. The imbalance of accumulation of these two metabolites may be highly indicative that organisms suffer oxidative stress [156, 157]. The redox state is determined in organisms assuming that $NADH/NAD^+$ can be accurately measured from the lactate/alanine ratio [158].

Therefore, the objective was to quantify relevant metabolites such as, glucose, lactate and alanine, and verify the metabolic and redox state of the cells after exposure to Hg. However, it was only possible to quantify two metabolites, lactate and alanine (fig. 13). Since concentration of glucose varies in response to environmental stress, the inability to quantify this metabolite might be explained by the fact of cells storing it in the form of glycogen [42]. This polysaccharide represents the readily storage form of glucose for most organisms and due to its complexity is not possible to be detected by ^1H NMR spectroscopy. The glycogen and lipids are the major energy storage in sea anemones [154]. Boury-Esnault and Doumenc [159], explain that glycogen levels increase in tissues of the *A. equina*, when induced an unfavorable physiology conditions, associated with tissue autolysis.

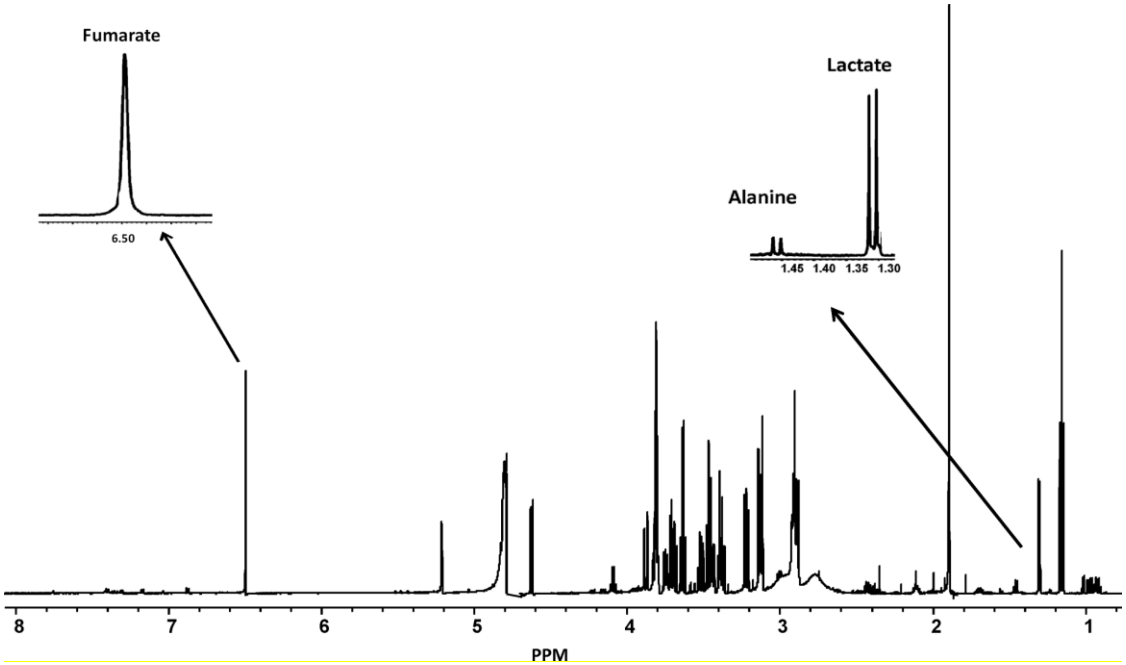

Figure 13. Representative ^{1}H NMR spectrum of pedal disc tissue extracts from a sea anemone *Actinia equina*. Representation of fumarate, alanine and lactate peaks. Fumarate is used as an internal reference to quantify metabolites in solution.

The table 11 shows the values of lactate and alanine concentrations, and the ratio between the two metabolites, in tissues extract of *A. equina*. The populations showed a high variability of responses after short-term exposure to Hg.

Table 11. Average of concentrations (mean ± SE) of the lactate and alanine metabolites (µmol g^{-1} dw), in tissue extracts of *Actinia equina*, and ratio of lactate/alanine after being exposed for 96h to artificial sea water and to 100 µg L^{-1} of Hg.

		Lactate (µmol g^{-1} dw)	Alanine (µmol g^{-1} dw)	lactate/alanine
S1	**Control**	0.415 ± 0.105	0.253 ± 0.048	1.659 ± 0.317
	Exposed	0.242 ± 0.008	0.682 ± 0.135	0.427 ± 0.093
S2	**Control**	1.275 ± 0.245	1.149 ± 0.172	1.258 ± 0.344
	Exposed	1.374 ± 0.413	0.334 ± 0.068	3.898 ± 0.707
S3	**Control**	0.590 ± 0.157	0.292 ± 0.040	2.005 ± 0.492
	Exposed	3.318 ± 0.629	0.230 ± 0.021	14.15 ± 2.275
S4	**Control**	1.792 ± 0.235	0.347 ± 0.079	5.706 ± 0.969
	Exposed	0.715 ± 0.302	0.528 ± 0.091	1.396 ± 0.556

Significant differences in the levels of lactate, alanine and in the ratio lactate/alanine were observed among organisms of *A. equina* collected at the four sampling sites. As well, significant differences were observed between treatments (control and Hg exposure) (table 12).

Table 12 Results of a two-way ANOVA testing for lactate, alanine and ratio of lactate/alanine, in the study of *Actinia equina* by sampled site and by treatments (control vs. exposed to Hg). The statistically different values are marked with asterisk (*) $p < 0.05$; (**) $0.05 < p < 0.01$; (***) $p < 0.001$. DF = degrees of freedom; SS = sum of squares; MS = mean of squares.

Sources of variation	DF	SS	MS	F	*p*
Lactate					
Treatment	1	0.0000680	0.0000680	0.000972	0.975
Site	3	2.444	0.815	11.654	**<0.001*** **
Treatment x Site	3	2.380	0.793	11.351	**<0.001*** **
Residual	32	2.237	0.0699		
Total	39	7.060	0.181		
Alanine					
Treatment	1	0.00101	0.00101	0.0369	0.849
Site	3	0.660	0.220	8.012	**<0.001*** **
Treatment x Site	3	1.311	0.437	15.916	**<0.001*** **
Residual	32	0.878	0.0275		
Total	39	2.850	0.0731		
Ratio lactate/alanine					
Treatment	1	0.000556	0.000556	0.00876	0.926
Site	3	3.247	1.082	17.035	**<0.001*** **
Treatment x Site	3	4.892	1.631	25.670	**<0.001*** **
Residual	32	2.033	0.0635		
Total	39	10.172	0.261		

The organisms of S1 showed a less accumulation of lactate (control and Hg exposure), than organisms of the others sites, but considerable high levels of alanine when exposed to Hg, in two treatments (fig. 14 and 15). The S2 population when exposed to Hg showed a significantly lower of alanine compared with control. The organisms of the S3 did not showed significant differences for alanine between treatments, while showing a sharp increase in lactate when exposed to Hg, when compared with control. Finally, the S4 population when exposed to Hg decreased the concentration of lactate when exposed to

Hg, but did not present statistical differences in concentration of alanine between treatments. Overall, two populations stand out when exposed to Hg: the S3 showed higher concentrations of lactate and S1 the highest concentration of alanine. The accumulation of lactate in high quantities, due to lack of metabolization, is often associated to metabolic stress as lactate does not follows degradation after glycolysis. This situation leads to a net acidification of the cell that can have deleterious effects on metabolism, particularly when long-term hypoxic or anoxic stresses are involved [160].

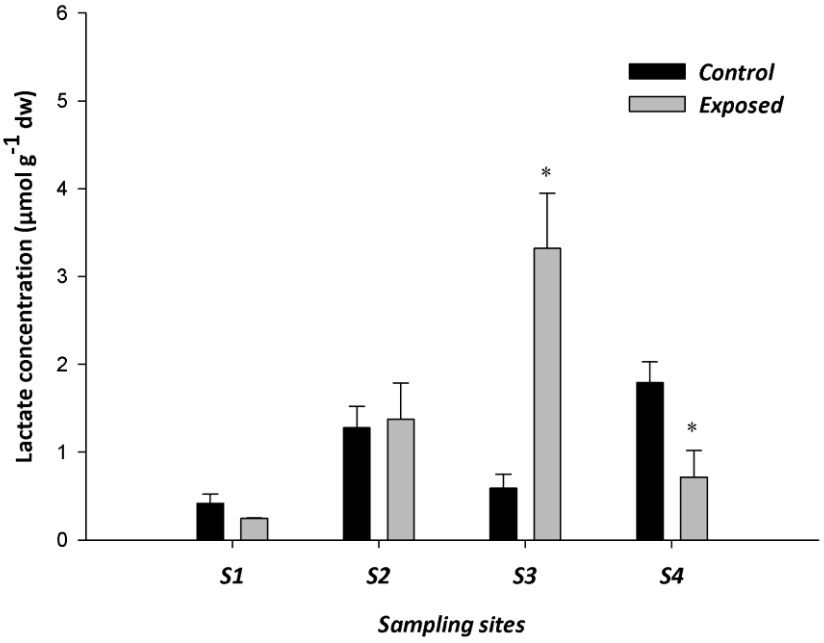

Figure 14. Average concentrations of lactate (µmol g^{-1} dw) (error bars represent standard error), obtained by NMR spectroscopy, in tissue extract of *Actinia equina* individuals, collected at the four study sites, after being exposed for 96h to artificial sea water and to 100 µg L^{-1} of Hg. The statistically differences between treatments (control and exposed to Hg), are marked with asterisk (*), $p < 0.05$.

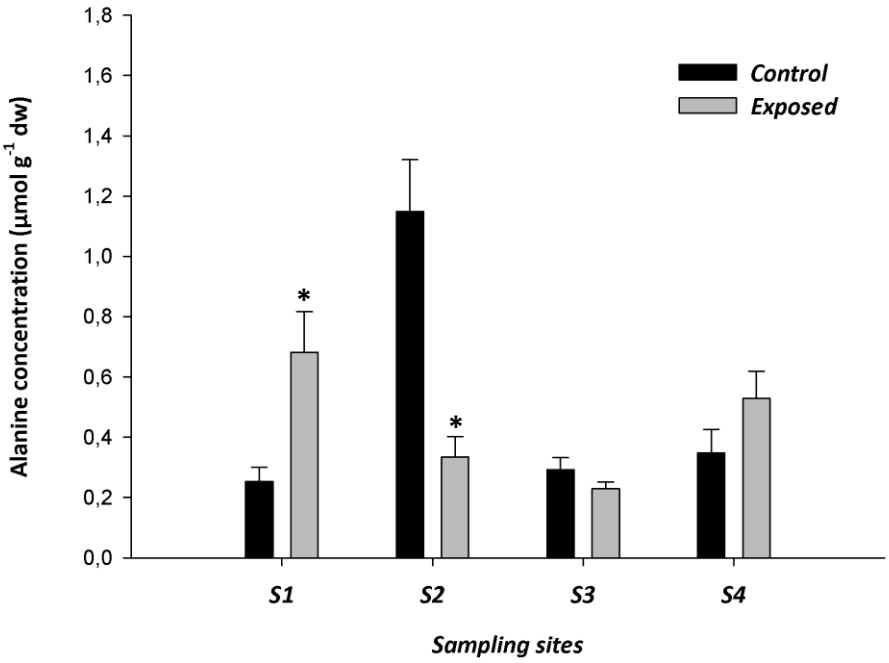

Figure 15. Average concentrations of alanine (µmol g^{-1} dw) (error bars represent standard error), obtained by NMR spectroscopy, in tissue extract of *Actinia equina* individuals, collected at the four study sites, after being exposed for 96h to artificial sea water and to 100 µg L^{-1} of Hg. The statistically differences between treatments (control and exposed to Hg), are marked with asterisk (*), $p < 0.05$.

It has already been shown that cytoplasmic NADH/NAD^+ redox potential exert important effects on energy metabolism. Because NADH serves as a cofactor for several enzymes, the NADH/NAD^+ ratio in the cytosol governs the activity of several enzymatic reactions of intermediary metabolism. Therefore, it is of interest to examine the factors that determine the redox potential and state of NADH/NAD^+ in the cytoplasm [161]. The cytosolic NADH/NAD^+ ratio can be accurately measured by the lactate/alanine ratio [158]. The results obtained for the lactate/alanine ratio, showed a high variability between populations, but presented statistically significance differences between control and exposure to Hg (fig. 16). The organisms of S1 showed a decreased in ratio when compared exposed to Hg with control. The organisms of S4 showed a similar behavior, but while the ratio of S1 decreased because the high levels of alanine, the S4 decreasing in ratio was due to a decrease in lactate. In organisms of S2 and S3 the results of the ratio lactate/alanine were different. The ratio of these populations increases in the exposed organisms, showing an inverse behavior of S1 and S4 populations. However, the ratio increased in S2 because of a high decrease in alanine while in S3 the increasing of the ratio is due to the high increase in lactate. Nevertheless, it was only possible in organisms

of the S1 and S4 verify the redox state of the cells using the ratio of lactate/alanine. These organisms (S1 and S4) showed a low NADH/NAD^+ ratio, both has low levels of lactate, it means that lactate is being spent, to maintaining redox balance.

The organisms of S3 present an increase of lactate in the tissues which indicates that cytosolic pyruvate is being converted to lactate. The exposure to Hg may lead to an increasing in glycolysis, which leads to an increase in NADH and therefore in an elevated NADH/NAD^+ ratio. To continuously supply the glycolysis new NAD^+ needs to be generated from other mechanisms, like for instance the conversion of pyruvate to lactate [162]. The cell under stressful conditions may not have capacity of response to the high accumulation of lactate, and leads acidification of cell. However, it was not possible to observe what occurred in alanine, so we cannot be verify the redox state of the cell.

In S2 is not possible to verify what occurred in the levels of lactate. The decrease in levels of alanine may be due to the fact of being converted to pyruvate for the Krebs cycle, but no inference could be done on ratio of NADH/NAD^+.

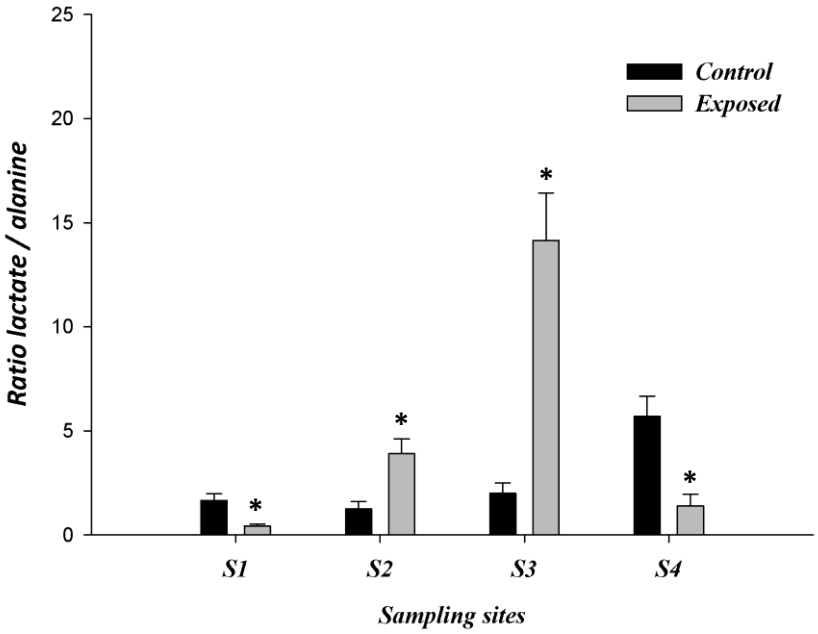

Figure 16. The ratio values of lactate/alanine (error bars represent standard error), obtained by NMR spectroscopy, computed for *Actinia equina* individuals, collected at the four study sites, after being exposed for 96h to artificial sea water and to 100 µg L^{-1} of Hg. The statistically differences between treatments (control and exposed to Hg), are marked with asterisk (*), $p < 0.05$.

3.5. Effects of exposure to mercury in *Actinia equina*

The results of bioaccumulation in the field, when compared with bioaccumulation in laboratory, were opposite. The organisms of S1 and S4, in the field bioaccumulate more Hg, and in laboratory were the organisms of sites S2 and S3. However, the results of LPO showed that organisms of S1 and S4 (sites with more contamination for Hg in the field), have more oxidative damage, probably reflects the damage due to the contaminated environment in which organisms live on.

In the data obtained by NMR-based metabolomics, the levels of ratio lactate/alanine reflect the data obtained in the bioaccumulation. The organisms that bioaccumulate less Hg in laboratory, organisms of S1 and S4, showed decreased of ratio lactate/alanine, probably because these organisms are less stressed than organisms of S2 and S3 sites.

Considering the background of contamination by Hg in the field, is possible to hypothesize about the behavior of the organisms after exposure to Hg in the laboratory. Organisms may have acquired resistance during long-term exposure to Hg in the field and this could be reflecting the response to short-term exposure to Hg in laboratory. Therefore, the results obtaining for Hg contamination in the field indicate that more contamination of Hg leads to the development of more resistance to the metal in the laboratory tests.

In conclusion, it was possible to confirm that short-term exposure to sublethal Hg had a significant influence on the levels of biological organization of organisms of *A. equina* collected at the four sampling sites.

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Chapter IV

1. Conclusion

Actually, the currently existing studies are sparse in relation to the distribution and localization of metals in cnidarians and this is due to the existence of very few data on the basic cellular compositions. The lack of information about the phylum makes it difficult to understand the responses of these organisms to adverse environments and also to interpret the responses, obtained in the present work of *A. equina* when exposed to the Hg.

In this work we conclude that Hg has effects in sea anemone *A. equina*, but we should take also into account the influence that levels of Hg found in the field may be have in the response of the organism to Hg short-term exposure in the laboratory. The presence of other contaminants in the field can also be difficult to interpret, as they may also interfere with the activity of the enzymes.

Nevertheless, the results obtained showed that organisms with accumulate less Hg in the field accumulate more Hg during the short-term exposure (S2 and S3). Concerning LPO, the organisms were accumulating more Hg in the field (S1 and S4) showed high levels of damage comparing with organisms of accumulate less Hg in the field (S2 and S3). Considering the background of contamination by Hg in the field, is possible to understand the behavior of the organisms in the laboratory. Since, this situation may be related with some resistance that organisms acquired with the exposure to a contamination in field. Generally, we have two reasons that shed light why organisms in long term may be resistant to a pollutant: individual organisms may have acquired a degree of tolerance by physiological acclimation during exposure to sublethal concentrations at some prior period of their lives, and populations may have evolved genetically based resistance, through the action of natural selection. However the first one does not confer tolerance to lethal concentrations upon offspring, who must also be pre-exposed to acquire it.

Despite some limitations that aroused from this work, should be borne in mind that some studies are preliminary in this species. However, it was possible observing different pattern of response when organisms were exposed to Hg. This characteristic, together with the fact that are easy to handle and maintain in laboratory, and the largest distribution of *A. equina* along the coastal zones of Atlantic meets the requirements to

use this species in ecotoxicological research. Notwithstanding, this species may be very promising for environmental monitoring studies.

1.1. Future work

Despite numerous studies on *A. equina*, few are those that are related to ecotoxicology. So, some future work that is likely to complement this done is:

1. Determination of lethal dose concentrations, to investigate the more realistic sublethal effects of trace metals;
2. Exploration the behavior of organisms in chronic tests will be useful in bioaccumulation tests using food contaminated, which will allow the determination effects of long term exposure of contaminant;
3. Investigation in detail the cellular levels where metals have effects and detects more biochemical changes resulting from exposure to metals;
4. To understood the oxidative stress probably the study of SOD and GR activity and the non enzymatic biomarkers GSH and glutathione oxidized (GSSG), are useful. These biomarkers can explain in detail what happens to organisms when exposed to trace metals;
5. Determination of Lactate dehydrogenase activity, that catalyzes the conversion of pyruvate and lactate;
6. And, in order to pursue this work it would be important to identify more metabolites that may provide the necessary information to understand how the species behaves in stressful conditions.